REMARKS

Claims 1-11 and 15-16 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over AMANN, et al. The Examiner was unpersuaded by Applicants previous argument that AMANN does not teach the use of a halide because AMANN teaches using "chloride peroxidase." Applicants explained that the presently claimed invention does not work unless a halide is present as well as a peroxidase and that a chloride peroxidase does not contain, a halide.

The Examiner was unpersuaded as it is allegedly "known in the art that a haloperoxidase ... is a peroxidase plus a halide or combination of halides" citing the abstract of Allen (US 6,503,507). Allen does not address the structure of chloride peroxidase. Mention of "halide:hydrogen peroxide (H2O2) oxidoreductase" in the abstract is simply another common name for chloride peroxidase and in no way indicates the presence of chloride. In fact, as stated by Allen, a halide or combination of halides is added to the solution.

Chloroperoxidase is an enzyme (protein) composed of amino acids and a heme center. It does not contain a halide. See for example Griffin, B.W. (1991) "Chloroperoxidase: a review." Peroxidases in Chemistry and Biology. Everse, et al. CRC Press, Boca Raton, vol. II, pp. 85-137 for physical properties of this enzyme. Therefore, as chloroperoxidase does not contain a halide, AMANN does not teach or suggest the present invention which includes a halide, and the rejection has been overcome.

In view of the foregoing, Applicant submits the Application is now in condition for allowance and respectfully requests early notice to that effect.

Respectfully submitted,

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Chapter 4

CHLOROPEROXIDASE: A REVIEW

Brenda W. Griffin

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I. INTRODUCTION

Only three heme-containing proteins are known to utilize H_2O_2 for oxidation of Cl^- to a reactive chlorinating species: chloroperoxidase from *Caldariomyces fumago*,[1-3] myeloperoxidase in neutrophils,[4-6] and a peroxidase in eosinophils.[7,8] Neidleman and Geigert, in a very thorough recent review of "biohalogenation" reactions, have enumerated many fungal sources of chlorinating activities, as established by isolation of halogenated products from the growth media of these species.[2] With few exceptions, the enzymes responsible for these chlorination reactions have not been purified and characterized. However, Geigert and coworkers recently reported the first example of a nonheme chloroperoxidase;[9] this enzyme, isolated from the fermentation of *Curvularia inaequalis*, contains both zinc and iron.[9] This review article will be concerned primarily with the hemeprotein chloroperoxidase isolated from *Caldariomyces fumago*, which has been extensively characterized by Hager and coworkers[1,10,11] since the first report on the enzyme appeared from this laboratory more than 25 years ago.[12] Both myeloperoxidase and the eosinophil peroxidase are reviewed in other chapters of this series.

Chloroperoxidase occupies a unique niche among hemeprotein enzymes; it is an extremely versatile catalyst, which exhibits, in addition to the halide oxygenation function, well-characterized dehydrogenation and H_2O_2-decomposing (catalatic) activities typical of other classes of hemeproteins.[1,2,10,11] Moreover, chloroperoxidase has many of the physical properties,[13-16] and certain oxygen transfer activities,[17-19] of the cytochrome P-450 class. A central theme in the study of hemeproteins has been the correlation of specific structural features with characteristic functions of distinct classes of these proteins. In this context, the diversity of chloroperoxidase catalytic activities appears quite anomalous. It is generally acknowledged that complete knowledge of the static structure of an enzyme in its resting state is inadequate to elucidate kinetic details of perhaps subtle structural and electronic changes in the molecule during its catalytic cycle. In the case of electron transfer reactions involving a mediator such as the heme group, this challenge is magnified by the intricate choreography of protein and prosthetic group that establishes the most favorable "path" of electron transfer, and, in some instances, concomitant oxygen atom transfer. This review will not be an exhaustive compilation of published data on chloroperoxidase, because some very excellent reviews of this enzyme have appeared recently.[1-3,20] Instead, we shall attempt to update and complement other reviews, with the goal of critically assessing current knowledge of the catalytic function of chloroperoxidase in chlorination reactions, the class of reaction most characteristic of this hemeprotein. In the spirit of this series, some speculation about possible reaction mechanisms will be made, where experimental data are lacking or incomplete, to stimulate further study of chloroperoxidase catalytic function.

II. BIOSYNTHESIS AND PHYSICAL PROPERTIES OF CHLOROPEROXIDASE

A. PHYSICAL PROPERTIES AND MOLECULAR BIOLOGY

Chloroperoxidase has been isolated in high yield from fermentation broths of the mold *C. fumago* and has been crystallized,[21] but only preliminary X-ray crystallographic data have been published.[22] Two of the three reported molecular species of chloroperoxidase have been characterized and shown to be true isoenzymes, i.e., they have the same amino acid composition and specific halogenation catalytic activity, but different carbohydrate content.[23] The major isozyme is a single subunit with molecular weight of 42,000 Da, determined by hydrodynamic measurements;[21] this value agrees well with the molecular weight of 40,500 Da computed from the amino acid composition (32,974) and carbohydrate content (approximately 7,500) of this isozyme.[23] The carbohydrate content (approximately 19%) of the major isozyme of chloroperoxidase is comparable to the carbohydrate content of horseradish peroxidase,[24] but greater than

that of hemeprotein peroxidases from mammalian sources, all of which are also glycoproteins.[2] Two asparagine residues of this isozyme, one present in the N-terminal chymotryptic peptide, were identified as the sites of *N*-glycosylation by high-mannose containing oligosaccharides.[23] In addition to significant amounts of mannose, this isozyme contains *N*-acetylglucosamine, galactose, and minor amounts of xylose and arabinose.[23] By contrast, the minor isozyme that was characterized contained much less mannose and no xylose or arabinose.[23] Apparently, heterogeneity of glycosylated chloroperoxidase molecules has been the primary problem in preparing crystals suitable for X-ray diffraction.[25] The blocked N-terminal residue has been shown to be pyrrolidone carboxylic acid, derived from rearrangement of a glutamic acid residue.[23] Since chloroperoxidase does not contain sialic acid, the differences in net charge of the three molecular species were attributed to partial deamidation of asparagine or glutamine residues.[23] Chloroperoxidase contains one mole of ferriprotoporphyrin IX, and is the only hemeprotein haloperoxidase known to also contain Mn^{2+}, at a variable mole ratio of 0.3 to 1.0.[10]

Recent studies have focused on control of expression of the chloroperoxidase gene by *C. fumago*.[26] When the fungus is grown on fructose as the sole carbon source, the enzyme is selectively induced and secreted into the medium in large quantities at approximately 90% purity.[26] Two different cDNA clones encoding parts of the chloroperoxidase gene have been isolated by modification of the mRNA priming procedure for constructing a cDNA library of *C. fumago*.[25,26] The technique of priming with a 29-base oligonucleotide complementary to mRNA of the protein greatly enriched the clones containing chloroperoxidase-specific sequences[26] compared to the method of using an oligo(dT)-primed cDNA bank.[25] The cDNA isolated by the former procedure was employed as a probe to investigate the control of secretion of the enzyme by the fungus under different experimental conditions.[26] It was demonstrated that both chloroperoxidase and chloroperoxidase-specific mRNAs are coordinately regulated by the carbon source available; with three different carbon sources, three effects were seen, namely, repression by glucose, induction by fructose, and an intermediate effect in the presence of glycerol.[26] It was proposed that these controls are mediated by the primary DNA structure of the transcriptional unit of chloroperoxidase, and that this system might be exploited for high-level expression and secretion of foreign gene products.[26]

Recently, the DNA sequence of the chloroperoxidase gene isolated within a 16.3-kilobase insert in the vector λEMBL3 and its immediate flanking regions was reported.[27] No introns and no significant homologies of the gene with known DNA sequences were found. One strong and two weaker initiation sites were identified with the general pattern of an adenine residue in the midst of a stretch of pyrimidines typical of eucaryotic transcription initiation sites.[27] Also, two sites with TATA box-like sequences were demonstrated to be approximately 100 basepairs upstream from the start of the transcription site, a feature that probably insures initiation of transcription at a single site.[27] The glutamic acid codon at the amino terminus is preceded by a 21-amino acid coding sequence, inferred to be a signal peptide that is cleaved during secretion of chloroperoxidase; this peptide has the requisite features, including a hydrophobic core, of known signal peptides.[23,25,27] Although the glutamine residue at the cleavage site of the putative signal peptide is unusual, the existence of an adjacent arginine residue allows the possibility of cleavage by a protease that recognizes a pair of basic residues; two examples of such processing were cited.[27] An earlier report of this laboratory noted that 25% of the arginine residues in chloroperoxidase are coded by the rare codon, AGG, in contrast to a low frequency of use of this codon (0.29%) for arginine residues in 25 different *Escherichia coli* genes examined.[25]

B. SIMILAR HEME STRUCTURES OF CHLOROPEROXIDASE AND CYTOCHROME P-450

When the optical spectra of various ferric and ferrous states of chloroperoxidase were characterized, quite unexpectedly many similarities between the enzyme and cytochromes P-450 were discovered.[13] It was particularly noteworthy that the carbon monoxide complex of

ferrous chloroperoxidase exhibits an absorbance maximum near 450 nm, the feature which distinguishes and confers the name of the cytochrome P-450 class of hemeproteins.[28,29] Additional similarities of various forms of chloroperoxidase and soluble cytochrome P-450 isolated from camphor-grown *Pseudomonas putida* have been established by use of sophisticated spectroscopic techniques that probe the electronic environment of the heme iron.[3]

Dawson has recently reviewed in depth the considerable amount of comparative spectroscopic data published for these hemeproteins.[3,20] The use of EXAFS spectroscopy proved to be particularly valuable, since this technique provided the first direct evidence for a sulfur atom occupying one of the axial ligand positions of the heme iron.[30,31] Analysis of the EXAFS data for five stable states of cytochrome P-450 and two states of chloroperoxidase by curve fitting procedures yielded values of the Fe-S distance ranging from 2.2 Å for the low spin ferric forms to 2.37 Å for the low spin ferrous-O_2 complexes.[30,31] These values agreed well with X-ray crystallographic data for model iron-porphyrin complexes with a thiolate (RS^-) ligand but were inconsistent with the larger Fe-S distance determined for a model complex with a thiol (RSH) ligand.[32] The recently-published X-ray crystal structures of low-spin (substrate-free) and high-spin (substrate-bound) ferric forms of the cytochrome P-450 isolated from camphor-grown *P. putida* have demonstrated a sulfur atom in the coordination sphere of the iron and confirmed the EXAFS-derived Fe-S distances.[33,34] These data provided convincing evidence for a proposal first made by Mason,[35] based on electron paramagnetic resonance studies of "model" complexes of myoglobin with sulfhydryl-containing ligands, that the unusual spectroscopic properties of cytochromes P-450 could be attributed to a cysteine-donated sulfur axial ligand rather than a histidine nitrogen, the axial ligand of horseradish peroxidase and the O_2-transport hemeproteins hemoglobin and myoglobin.[36] The available evidence is most consistent with ligation of cysteine as its ionized RS^- form, rather than the RSH species, at the axial position of the heme iron of both chloroperoxidase and cytochrome P-450.[20] Dawson has concluded that the different Fe-S distances observed by EXAFS for the high-spin ferric forms of cytochrome P-450 (2.23 Å) and chloroperoxidase (2.30 Å) cannot be attributed to uncertainty in the data (±0.02 Å) and may account for certain differences in the physical properties of the enzymes.[20] The amino acid sequence of chloroperoxidase revealed,[23] and the nucleotide sequence of the gene confirmed,[27] the presence of only three cysteine residues out of 300 amino acids. One of the cysteine residues, Cys_{87}, is in a region that displays limited homology with the peptide region of cytochrome P-450 which contains the cysteine axial ligand, Cys_{357} in the case of the *P. putida* enzyme.[33] The proposal that Cys_{87} is the axial ligand of chloroperoxidase must be confirmed by other experiments, such as site-directed mutagenesis. The two remaining cysteine residues of the protein were presumed to form a disulfide bond,[23] since earlier experiments had shown that chloroperoxidase has no free sulfhydryl groups.[37]

The other axial ligand of these ferric hemeproteins depends on the spin state: in the case of the high-spin (S = 5/2) species, the sixth ligand position of each enzyme is thought to be vacant.[20] The X-ray crystallographic data for camphor-bound high-spin cytochrome P-450 from *P. putida* are consistent with this interpretation, but cannot eliminate the possibility that loosely bound, disordered H_2O molecules which do not exchange rapidly with bulk solution water occupy the protein channel by which substrate gains access to the active site.[33] Native low-spin (S = 1/2) ferric cytochrome P-450 has been shown to have a H_2O (or possibly OH^-) ligand with protons that exchange rapidly with the bulk solution H_2O,[38] quite consistent with the X-ray crystal structure of this form of the protein.[34] It can be inferred from these data that, in the absence of substrate, solvent H_2O molecules have greater access to the substrate-binding channel and to the heme iron.[34] The consequence is that on average a H_2O molecule (with short residence time) occupies the other axial ligand position of low-spin cytochrome P-450.[38] The iron of high-spin pentacoordinate cytochrome P-450 is displaced 0.44 Å out of the plane of the porphyrin structure.[34] This "puckering" of the heme plane has been observed for other high-spin hemeproteins for which X-ray crystallographic data are available.[39] However, substrate-free

low-spin cytochrome P-450 has the iron similarly displaced from the plane of the porphyrin.[39] Thus, this structural feature probably derives from coordination of the cysteinate ligand, since it appears not to be significantly altered by a spin-state change induced by substrate binding near the heme group.[39] Low-spin chloroperoxidase, produced from the native high-spin species by lowering the temperature below 200 K or increasing the pH above 7.0, is thought to have a histidine sixth ligand.[14,15] This is consistent with evidence for an acidic amino acid residue with pK_a near 5.5 that participates in binding of exogenous ligands at the displaceable coordination position of ferrous chloroperoxidase.[15,40]

III. REACTIONS CATALYZED BY CHLOROPEROXIDASE

A. OVERVIEW

As mentioned in Section I, chloroperoxidase is perhaps the most versatile of hemeprotein catalysts. The enzyme catalyzes, at quite respectable rates, four distinctly different types of reactions with H_2O_2 or certain other peroxidic agents as the source of oxidizing equivalents: halogenation of organic compounds with Cl^-, Br^-, or I^- as the halogen source, dehydrogenation, H_2O_2 decomposition, and oxygen transfer to certain inorganic and organic compounds.[1,2] These reactions are illustrated in general form below:

Halogenation: $$AH + H_2O_2 + X^- + H^+ \rightarrow AX + 2\,H_2O \quad (1)$$

where $X^- = Cl^-$, Br^-, or I^-;

Dehydrogenation: $$2\,BH + H_2O_2 \rightarrow B\text{–}B + 2\,H_2O \quad (2)$$

Decomposition of H_2O: $$2\,H_2O_2 \rightarrow O_2 + 2\,H_2O \quad (3)$$

Oxygen insertion: $$R + H_2O_2 \rightarrow RO + H_2O \quad (4)$$

Representative reactions of each class will be considered before proceeding to a discussion of reaction mechanisms.

Neidleman and Geigert[2] presented a comprehensive listing of chloroperoxidase-catalyzed halogenation reactions in their definitive book on biohalogenation. They noted that halogen substitution, catalyzed by chloroperoxidase and other haloperoxidases, typically occurs at a carbon atom having an "activated" carbon-hydrogen bond.[2] The presence of a double bond, a phenyl group, and, to a lesser extent, certain heteroatoms is known to activate hydrogen atoms on adjacent carbons.[41] Homolytic cleavage of activated C-H bonds requires less energy because the resultant organic radical species can be stabilized by delocalization of the unpaired electron; this enhances the rate of hydrogen atom abstraction from such species by less stable radicals.[41] The few oxygen-insertion reactions on carbon catalyzed by chloroperoxidase (Equation 4) appear to be similarly facilitated by an activated hydrogen atom or easily abstracted electron.[17,18]

Certain critical features of the oxygen insertion activities of chloroperoxidase are quite analogous to the monooxygenase function of cytochromes P-450, the general equation for which is[28,29]

$$R + O_2 + NAD(P)H + H^+ \longrightarrow RO + H_2O + NAD(P)^+ \quad (5)$$

Two electrons originating in a reduced pyridine nucleotide are transferred, via an associated electron transfer system consisting of one or more proteins, to cytochrome P-450. One electron

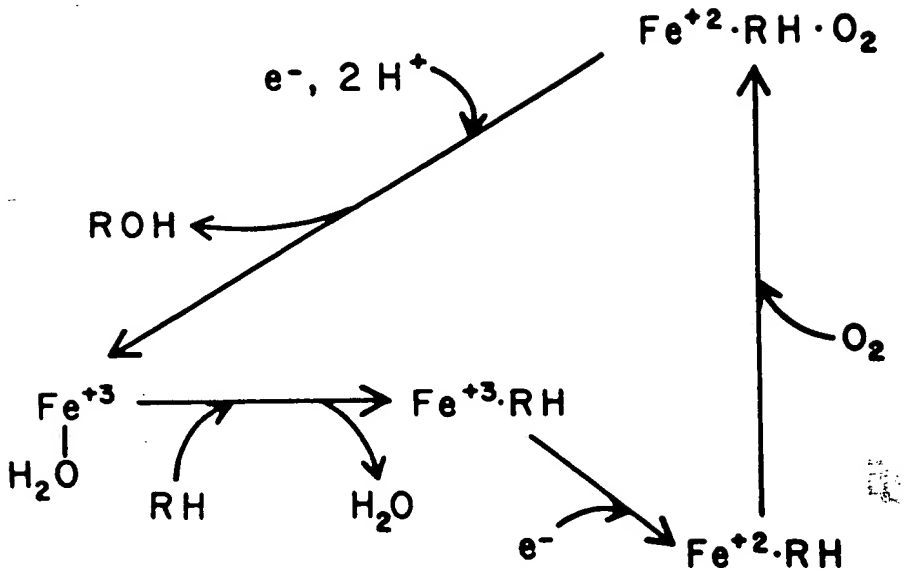


FIGURE 1. Catalytic cycle of cytochrome P-450. A minimal catalytic cycle is shown, comprising four distinct well-characterized forms of cytochrome P-450. The vertical positions of the different oxidation states of the enzyme correspond in a very qualitative manner to differences in their energy content; the substrate-bound ferrous and ferrous-dioxygen forms define the lower and upper limits, respectively, of this energy scale. The catalytic "triangle" is initiated by binding of the oxygen acceptor substrate (RH) to the resting ferric enzyme (lower left), which displaces the exchangeable H_2O (or OH^-) ligand. One-electron reduction of this complex produces the ferrous RH complex, which binds O_2 reversibly. The rate-limiting step of the cycle, transfer of an electron to the ferrous-dioxygen complex, yields product. The number of protons and the point(s) at which they enter the cycle are uncertain; at least one proton would be required for OH^- formation.

is transferred prior to, and the second electron subsequent to, binding of O_2 by the enzyme-substrate complex (Figure 1).[42,43] During catalysis, the enzyme-bound O_2 molecule becomes reduced to an oxidation state formally equivalent to that of H_2O_2. However, this two-electron reduced oxygen species has only a transient existence, since it must be sufficiently activated to bring about stereospecific oxygenation of R to RO.[44,45] One oxygen atom derived from O_2 becomes incorporated into RO, and the second oxygen atom is reduced by two electrons from the reduced pyridine nucleotide.[42,43] Peroxidases, in general, cannot catalyze the analogous reaction, that is, stereospecific insertion of an oxygen atom derived from H_2O_2 into the electron donor. Indeed, cytochrome P-450 cannot use H_2O_2, organic hydroperoxides, or other oxygen donors very efficiently for oxygen insertion into substrates.[46] There are only a few reactions of cytochrome P-450 with partially reduced oxygen-containing oxidants for which the oxygen atom incorporated into the reaction products has been conclusively established to originate in the oxidant.[47,48] Moreover, compared to the true peroxidases, cytochromes P-450 are ineffective catalysts of any reaction of H_2O_2, including dehydrogenation reactions.[46] Since cytochromes P-450 do not catalyze halogenation reactions, the oxygen insertion reaction appears to be the only reaction type of the four reactions, Equations 1 to 4, which is catalyzed to any significant degree by both cytochromes P-450 and chloroperoxidase. An important distinction between the two enzymes, however, is that certain isozymes of cytochrome P-450 can catalyze hydroxylation of hydrocarbons containing no activated hydrogen atoms,[28,49,50] in contrast to an apparent requirement for activated hydrogens on halogen or oxygen acceptor substrates of chloroperoxidase.[2,17,18]

Long before the axial ligand of cytochrome P-450 was identified as a cysteinate residue, it had been assumed that the unusual coordination of the heme group must be critical for the

monooxygenase activity of this enzyme: cleavage of molecular O_2 such that one oxygen atom is inserted into an organic substrate and the second oxygen atom is reduced to H_2O.[28,42,43] One aspect of this reaction that has received little attention is the ultimate disposition of the energy contained in the oxidant: a part of the energy that would be released as heat by four-electron oxidation of O_2 is, in fact, conserved through formation of a carbon-oxygen bond in the product. In terms of energy conservation, and the requirement for associated electron transport proteins, the monooxygenase function of cytochrome P-450 is somewhat analogous to mitochondrial oxidative phosphorylation, the compartmentalized, highly-regulated biochemical pathway by which a part of the energy produced by the four-electron reduction of O_2 is coupled to the synthesis of high-energy phosphate bonds.[51] However, with cytochrome P-450, the transfer of oxidizing equivalents and the energy-requiring bond-making process occur at the same active site via transfer of an "activated" oxygen atom from the enzyme to a carbon of the acceptor substrate.[28,49,50] The analogous reaction of chloroperoxidase is considered to be the transfer of an oxygen atom to Cl^-, which couples energy derived from reduction of the oxidant to formation of the O-Cl bond. The product of Cl^- oxygenation, HOCl, is a meta-stable species, which, upon dissociation from the enzyme, can react nonenzymatically with various components in the system.[2,52-54] The probability of reaction of HOCl with a "substrate" to yield a stable chlorinated product is increased if the compound is present in large excess and contains an activated carbon-hydrogen bond. If such compounds are unavailable, the energy of HOCl will be rapidly dissipated by reaction with other components in the system, including H_2O_2 and the enzyme, which will terminate the reaction.[10,53]

It is proposed that the function associated with the unusual cysteinate heme ligation of both chloroperoxidase and cytochrome P-450 is reductive cleavage of the O-O bond of the oxidant such that a significant fraction of the energy produced is conserved as a new chemical bond with relatively high energy: O-X^-, in the case of chloroperoxidase, and O-C in the case of cytochrome P-450. Since the substrates oxygenated by these hemeprotein enzymes typically have unfavorable redox potentials, each enzyme must facilitate a specific "activation" of an oxygen atom for the bond making process. Such oxygen-atom transfer reactions are expected to have greater steric and electronic constraints than hemeprotein-catalyzed dehydrogenation reactions, which involve transfer of relatively activated single electrons or hydrogen atoms from the substrates to oxidized heme species with highly delocalized electrons. By contrast, the efficient oxygenation of a compound requires a highly specific interaction between the compound and the heme oxygen atom donor species. If the proper orientation of the acceptor substrate cannot be achieved at the enzyme active site in the decay period of the transient heme oxygen donor, then the "activated" oxygen atom will undergo other less specific reactions, some of which probably result in inactivation of the enzyme.

It appears, however, that cysteinate ligation of an iron-protoporphyrin IX group is not the only type of heme structure that satisfies the functional requirement of chloroperoxidase with regard to Cl^- oxygenation, since the Cl^--oxygenating enzyme myeloperoxidase contains two identical heme groups that are completely different from the heme of cytochrome P-450 and chloroperoxidase. This simple analysis of the energetics of the reactions catalyzed by cytochromes P-450 and chloroperoxidase lays the foundation for a general hypothesis, to be described later, for the functional basis of similarities of the immediate heme environments of cytochromes P-450 and chloroperoxidase. It is clear that a detailed knowledge of the heme structure and its role in catalysis will not be sufficient to understand the great diversity and specificity of oxygenation reactions catalyzed by cytochrome P-450 isozymes. The active site protein differences among these isozymes will be defined only by X-ray crystallography. It appears likely that specific amino acid substitutions in the vicinity of the heme group control not only the specificity of binding of organic compounds to the heme and protein components of the active site, but also the detailed path of the oxygen atom transfer reaction of individual cytochrome P-450 species.

B. CHLORINATION REACTIONS OF CHLOROPEROXIDASE: GENERAL ASPECTS

The variety of chemical structures which are acted upon by the haloperoxidases as a class was probably not fully appreciated prior to the exhaustive review by Neidleman and Geigert, who documented many novel reactions of chloroperoxidase studied in their own laboratory.[2] The examples cited by these authors generally depict chloroperoxidase-catalyzed chlorination reactions; however, in most cases, analogous reactions of this enzyme occur with Br^- or I^- as the halide donor.[2] A few generalizations about these enzymatic halogenation reactions can be made to set the stage for the subsequent discussion of mechanism. First, as mentioned in Section I, halogen substitution generally occurs at a carbon atom containing an activated hydrogen atom.[2] A second point emphasized by Neidleman and Geigert[2] is that these enzymatic halogenation reactions do *not* display characteristics typical of most enzymatic reactions, such as reversibility, narrow substrate range, and high product selectivity. We note that reversibility is not a definitive criterion for enzymatic reactions; for example, reactions catalyzed by haloperoxidases, oxidases, and oxygenases, involving O_2 or H_2O_2 as oxidant, are quite exothermic and, thus, effectively irreversible under physiological conditions. However, it is quite unusual for the pH optimum and the product selectivity of enzymatic reactions to depend upon the concentrations of both substrates, as occurs with haloperoxidases.[2]

Finally, a consideration of standard redox potentials of the half reactions for reduction of H_2O_2 and for oxidation of the various halides[55] reveals the thermodynamic constraints on these reactions, which must necessarily translate into constraints on the structures and reactivities of the different enzymes that can oxidize the respective halide anion.

Half – reaction	E°, Volts	
$2F^- \longrightarrow F_2 + 2e^-$	−3.06	(6)
$H_2O_2 + 2H^+ + 2e^- \longrightarrow 2\ H_2O$	+1.77	(7)
$2\ Cl^- \longrightarrow Cl_2 + 2e^-$	−1.36	(8)
$2\ Br^- \longrightarrow Br_2 + 2e^-$	−1.07	(9)
$2\ I^- \longrightarrow I_2 + 2e^-$	−0.54	(10)

It is clear that the energy released by reduction of H_2O_2 is insufficient to oxidize F^-, consistent with the failure of any known haloperoxidase to catalyze directly the H_2O_2-dependent oxidation of F^- to a fluorinating species. Moreover, the enzymes which can catalyze chlorination reactions can generally catalyze analogous bromination and iodination reactions.[2] On the other hand, "bromoperoxidases", such as lactoperoxidase, can utilize only Br^- and I^-,[56] whereas "iodoperoxidases" such as thyroid peroxidase, function only in catalysis of iodinations.[57,58] Since the reduction of H_2O_2 produces sufficient energy to oxidize Cl^-, Br^-, or I^-, the "specificities" of these enzymes for halide anion appear to reflect their differing abilities to utilize efficiently the energy generated by reduction of H_2O_2 for productive oxidation of halides. A simplistic explanation is that the few hemeproteins which can catalyze Cl^- oxidation by H_2O_2 are more efficient than iodoperoxidases at coupling the energy of the oxidant to halide oxidation. Although the actual half-cell potential for oxidation of each halide anion by H_2O_2 will depend on the experimental conditions, such as pH and reactant concentrations, for a set of specific experimental conditions, the half-cell potential for oxidation of any halide will differ from the standard half-cell potential by the same absolute amount. Thus, it seems likely that the significant differences among the haloperoxidases with respect to halide specificity reflect major differences in the reactivities,

and, by inference, in the structures of the oxidized enzyme species which directly oxidize the halide. Those enzymes which can oxidize Cl^- must produce an intermediate with a more positive half-cell reduction potential than those which can oxidize only Br^- and/or I^-.

C. CHLORINATION REACTIONS CATALYZED BY CHLOROPEROXIDASE

We have adopted the classification system of Neidleman and Geigert[2] in this brief discussion of chlorination reactions catalyzed by chloroperoxidase. For more detail, the reader is referred to their excellent review.[2] A variety of alkenes, both small and large molecules, can be converted to alpha, beta-halohydrins by a chloroperoxidase-containing halogenation system.[59,60] However, functional groups on the alkene and the halide concentration will alter the final products. For example, dihalide products (homogeneous or heterogeneous) are produced in the presence of very high concentrations of one or more halides.[61-63] The chloroperoxidase-catalyzed chlorination of alkyne substrates produces alpha-haloketones, with mono or di-halide substituents, depending on halide concentration in the reaction.[64] The enzymatic chlorination of a few cyclopropanes has been studied and shown to yield alpha, gamma-halohydrins.[64] Chloroperoxidase and other haloperoxidases catalyze halogenation of diverse aromatic compounds, including anilines, phenols, and heterocyclics.[65-68] These easily oxidized compounds are also good substrates for H_2O_2-dependent dehydrogenation by these same enzymes. The latter reactions compete with halogenation when halide anion is limiting or depleted.[11] Neidelman and Geigert noted that the reactivity of beta-diketones, such as monochlorodimedone (MCD), the standard halogen acceptor substrate used to assay haloperoxidase activity, is a function of enol content of the substance under the reaction conditions.[2] Thus, MCD, which exists almost completely as the enol form, is much more reactive than 2-heptanone, which contains only minor amounts of the enol form.[69,70] The diversity of chemical structures of beta-diketones chlorinated by this enzyme is great and includes polycyclic compounds such as steroids.[71] The halogenation of beta-ketoacids results in their decarboxylation.[12] For most of these reactions, similar products are formed if Br^- or I^- is substituted for Cl^-, but the stability of the different halogenated analogs may vary.[65]

The oxidation of sulfur-containing compounds by chloroperoxidase can take various routes. In the presence of chloride, thiols are converted to the unstable sulfenyl chlorides, which can undergo further reaction to disulfides or sulfonic acids, depending upon the availability of excess thiol or OH^-.[72] Haloperoxidases will also oxidize disulfides and alkyl sulfides to sulfoxides in the presence of halide anion, which is a Cl^--dependent oxidation reaction.[73] Haloperoxidases catalyze the conversion of amines to unstable haloamines, which undergo deamination and decarboxylation in the absence of other components.[74] The generation of chloramines from amines normally present in biological systems by myeloperoxidase in stimulated neutrophils has been studied extensively by Thomas and coworkers and by Weiss' laboratory.[75,76] Most of the chloramines formed, including NH_2Cl (the product of NH_4^+ chlorination), are diffusible, reactive lipophilic products with variable cytotoxic and microbicidal activities.[77] However, the product of taurine chlorination is a relatively stable hydrophilic product.[75,78,79] Although chloramine formation has been most thoroughly characterized with myeloperoxidase, presumably chloroperoxidase functions similarly.

Finally, it has been reported that chloroperoxidase catalyzes the Cl^--dependent conversion of NAD(P)H to an intermediate which is oxidized by the enzyme and H_2O_2 to a second species.[80] Both reactions went to completion with stoichiometric H_2O_2 and yielded products distinct from the respective oxidized pyridine nucleotide.[80] Based on incorporation of $^{36}Cl^-$ into the reaction product and other experimental data, it was proposed that NAD(P)H is chlorinated and then oxidized, with retention of the Cl atom, by this system.[80] These results were consistent with earlier data demonstrating that myeloperoxidase catalyzes the reaction of NADH to unidentified product(s) inactive with several dehydrogenases.[81] The stepwise chlorination and oxidation of NAD(P)H to identical products could also be effected by adding aliquots of HOCl, to a total

amount approximately twice the molar concentration of NAD(P)H.[80] Related to these observations, Fridovich's lab[82] recently reported a biphasic inactivation of bovine liver catalase by NH_2Cl. NADPH, which is tightly bound to this particular catalase, was shown to be essential for the rapid phase of inactivation.[82] Since NH_2Cl converted free NADPH to a species distinct from $NADP^+$, it was proposed that a similar reaction occurred between NH_2Cl and catalase-bound NADPH· the product was presumed to be a chlorinated pyridine nucleotide which inactivated catalase by chlorinating the heme group or amino acid residues near the heme.[82]

The experiments cited and many others support the idea that chloroperoxidase, like myeloperoxidase, produces a very reactive, freely diffusible chlorinating agent with the properties of HOCl. Subsequent reactions of HOCl with components of biological systems may produce chlorinated, diffusible species sufficiently reactive to propagate neutrophil-initiated damage to cellular components. Many laboratories have conducted comparative studies of chlorination or oxidation of various compounds by both a chloroperoxidase system and HOCl. The results have generally shown the two systems to be indistinguishable. Where differences between the two chlorinating systems have been observed, it would appear that the *dynamics* of enzymatic formation of HOCl and of its subsequent reactions in systems containing H_2O_2 and Cl^- were not strictly mimicked by the analogous chemical reaction with HOCl. Additional evidence for free HOCl as the halogenation agent generated by chloroperoxidase will be presented later. However, it seems appropriate to quote the conclusion drawn by Neidleman and Geigert on this issue: "Although this controversy as to whether HOX or Compound EOX [of haloperoxidases] is the key halogenating intermediate will continue, it is safe to say that all products produced and *all* product ratios obtained (whether geometric or stereo) by haloperoxidase-catalyzed halogenations can be explained by hypohalous acid chemistry."[2]

D. OXIDATION REACTIONS CATALYZED BY CHLOROPEROXIDASE

Chloroperoxidase utilizes H_2O_2 for the oxidation of classical peroxidase substrates, including ascorbate, guaiacol, and pyrogallol.[11] With any peroxidase catalyst, these reactions involve transfer of single electrons from the electron donor substrate to one or more higher oxidation states of the hemeprotein resulting from interaction of the enzyme with H_2O_2.[83] Radical intermediates produced from the electron donor molecules dissociate readily from the enzyme and, if sufficiently stable, may accumulate to concentrations detectable by EPR spectroscopy.[84] The stable products of these reactions are characteristic of the aqueous solution free radical chemistry of the individual species. Indeed, the product profile may vary considerably, depending upon the experimental conditions, such as rate of radical generation, molar ratio of H_2O_2 to electron donor, pH, and presence of O_2 or other redox-active substances, including low levels of adventitious transition metal ions.[85,86]

Another reaction type catalyzed by chloroperoxidase and other peroxidases is *N*-demethylation:[87,88]

$$H_2O_2 + R\text{–}N\text{–}R' \rightarrow R\text{–}N\text{–}R' + H_2CO + H_2O \qquad (11)$$

Certain isozymes of cytochrome P-450, with their associated electron transport proteins, can catalyze analogous *N*-demethylation reactions in which O_2 and NADPH supply the partially reduced oxygen species.[89] The cytochrome P-450 reaction had been generally considered to proceed by direct insertion of an oxygen atom derived from O_2 into a C-H bond of the *N*-methyl group, generating an unstable carbinolamine which spontaneously decomposed to formaldehyde and the amine.[90] There is now convincing evidence that most hemeprotein-catalyzed *N*-demethylation reactions proceed by two-electron dehydrogenation of the substrate followed by addition of OH^- to yield the carbinolamine:[50,88,91]

$$\begin{array}{l} \overset{-e^-}{\rightarrow} \quad \overset{+}{\cdot} \quad \overset{-H^+}{\rightarrow} \quad \overset{+}{} \quad \overset{OH^-}{\rightarrow} \\ R\text{–}N\text{–}R' \rightarrow R\text{–}N\text{–}R' \rightarrow R\text{–}N\text{–}R' \rightarrow R\text{–}N\text{–}R' \rightarrow R\text{–}N\text{–}R' + H_2CO \\ \quad | \qquad\quad | \qquad\quad | \qquad\quad | \qquad\quad | \\ \quad CH_3 \qquad CH_3 \qquad CH_2 \qquad H_2COH \qquad H \end{array} \tag{12}$$

With *N*-methyl compounds that yield relatively stable free radical species, i.e., aminopyrine, significant levels of the radical may accumulate in solution before it loses an electron or reacts nonenzymatically by other routes, for example, dismutation or coupling with an identical radical or addition of O_2.[88] In some cases, with very unstable radical intermediates and/or with two electron enzyme oxidants, the dehydrogenation and/or hydrolysis steps of Equation 12 may occur at the enzyme active site prior to release of the carbinolamine product. In most cases, the carbinolamine loses formaldehyde readily. Consequently, the origin of the carbinolamine oxygen atom, whether derived from H_2O or H_2O_2 (or O_2 in the case of cytochrome P-450 catalysis) is indeterminant due to rapid exchange of the formaldehyde oxygen atom with H_2O.[92]

The *N*-demethylation of *N,N*-dimethylaniline by ethylhydroperoxide catalyzed by chloroperoxidase has been characterized with respect to steady-state kinetics[87] and deuterium isotope effects, by use of substrate with fully deuterated methyl groups.[93] Very similar, significant deuterium isotope effects were observed for both horseradish peroxidase and chloroperoxidase.[93] The data were consistent with transfer of a deuterium atom (instead of an electron) from the substrate to the enyzme, release of product from the enzyme either as the iminium cation or the carbinolamine (Equation 12), and subsequent displacement of the deuterium by ethylhydroperoxide at the start of the next catalytic cycle.[93] Finally, there is limited evidence for halide-dependent *N*-demethylation reactions catalyzed by chloroperoxidase: in the case of *N,N*-dimethylaniline, Cl^- stimulated the reaction supported by various peroxides only in the low pH range,[93] consistent with the known pH dependence of chloroperoxidase-catalyzed production of an HOCl-like species.[10,94] Also, *N*-demethylation of 4-haloantipyrine was demonstrated to occur with both HOCl and a chloroperoxidase halogenating system.[52] However, the release of formaldehyde from this compound required a three- to four-fold excess of oxidant (HOCl or H_2O_2), indicating competition for the oxidant by either the secondary amine product of the reaction or by the phenyl ring of the substrate/product.[52] In a very detailed study, Sayo et al.[95] compared the reaction of *N,N*-dimethyl-*p*-anisidine with HOCl, with a myeloperoxidase-H_2O_2 system in the absence or presence of Cl^-, and with a horseradish peroxidase-H_2O_2 system. The product distribution was somewhat different in each system, quite characteristic of the involvement of free radicals. However, they concluded that the radical cation of the substrate was the first oxidation product in all systems. Subsequent reactions of this species, whether via Equation 12 to produce formaldehyde or other routes, depended upon the dynamics and reactive components of each system.[95] The radical cation appeared to be particularly unstable in the presence of HOCl.[95] While the data of Sayo et al.[95] and the *N*-demethylation of 4-haloantipyrine[52] support the generality of the radical mechanism of *N*-demethylation reactions, Equation 12, they also demonstrate quite convincingly the complexity of reactions occurring in such systems.

Chloroperoxidase also catalyzes two other dehydrogenation reactions that are typical of catalases but not peroxidases: oxidation of ethanol to acetaldehyde[11] and H_2O_2 decomposition (Equation 3).[11,53] Ethanol oxidation by both chloroperoxidase and catalase appears to proceed by concerted transfer of two electrons from the substrate to the respective compound I enzyme species. Compound I (Section IV.A.2) is the two-electron oxidized species resulting from interaction of H_2O_2 with the respective resting ferric enzyme.[11,96] Although there is no evidence for participation of a radical species of ethanol in the enzymatic reactions, the possibility that a transient enzyme-bound radical is formed cannot be excluded. Regarding the H_2O_2-decomposing (catalatic) activities of chloroperoxidase and catalase, two important distinctions between the enzymes have been noted. The first concerns the origin of the oxygen atoms in O_2 produced in the reaction: with catalase, both oxygen atoms in O_2 arise from the same molecule of H_2O_2.[96]

This result is readily explained as a two-electron dehydrogenation of one H_2O_2 molecule by catalase compound I. With chloroperoxidase, the question of the origin of the oxygen atoms in O_2 evolved during catalatic activity of the enzyme was addressed by experiments with *m*-chloroperoxybenzoic acid doubly labeled with ^{18}O.[97] Although peroxidases and catalase can be oxidized to their compound I species by ethylhydroperoxide and peroxy acids, only chloroperoxidase is able to catalyze the decomposition of these oxidants to O_2 and the respective alcohol or acid product.[97] The chloroperoxidase catalyzed decomposition of ^{18}O-labeled *m*-chloroperoxybenzoic acid resulted in "scrambling" of the oxygen atoms in the evolved O_2, that is, the atoms originated in two different molecules of the peroxy acid. It might be concluded from this result that chloroperoxidase compound I contains an oxygen atom derived from one molecule of the peroxy acid which can be transferred to another molecule of the peroxy acid, although other explanations may be possible. Apparently, the analogous experiment has not been performed with ^{18}O-labeled H_2O_2 and chloroperoxidase to determine if the oxygen atoms of O_2 produced are "scrambled". However, the unique ability of chloroperoxidase to catalyze what appears to be transfer of an oxygen atom to a peroxy acid suggests that the chloroperoxidase species active in decomposition of the peroxy acid has quite different properties from compound I species of catalase and peroxidases, which have only a few poorly characterized oxygen transfer activities.

A second important distinction between catalase and chloroperoxidase as catalysts of H_2O_2 decomposition relates to the effect of halide on the reaction. Chloride and bromide have no significant effect on H_2O_2 decomposition by catalase,[98] but stimulate the catalatic activity of chloroperoxidase approximately ten-fold.[11,53] In a detailed study, the halide dependence of the chloroperoxidase reaction was shown to be very similar to the stimulation of O_2 evolution in acidic mixtures of HOCl and H_2O_2 by Cl^- and Br^- (but not I^- or F^-).[53] Similar effects of added halide anion on oxidation of H_2O_2 by both the chemical and enzymatic halogenating systems were attributed to formation of the reactive X_2 species in equilibrium with H^+, X^-, and HOX. The acceleration of O_2 evolution by X^- in both systems results from faster oxidation of H_2O_2 by X_2 than by HOX. Moreover, Br^- was effective at lower concentrations than Cl^- in both systems. Indeed, Br^-, at rather large concentrations, was shown to markedly stimulate the negligible rate of H_2O_2 decomposition catalyzed by horseradish peroxidase, but very high Cl^- concentrations had no effect in this system.[53] These data suggested that both chloroperoxidase and horseradish peroxidase can produce an HOBr-like species, which, in the presence of excess halide in these acidic solutions, gives rise to Br_2 at concentrations sufficient to accelerate H_2O_2 oxidation. Horseradish peroxidase requires high concentrations of Br^- for this activity and is inactive with Cl^-, whereas chloroperoxidase can oxidize Br^-, at low concentrations, as well as Cl^- at much larger concentrations.[53]

In the course of these studies,[53] it was observed that Cl^- stimulation of chloroperoxidase catalatic activity depended upon the source of the Cl^-: moderate concentrations of an ultra pure grade of Cl^- actually inhibited O_2 evolution, whereas very high concentrations stimulated the rate. These results were attributed to a low, variable Br^- contamination of most alkali chlorides.[53] The addition of a very low amount of Br^- to the Cl^--inhibited catalatic reaction of chloroperoxidase stimulated O_2 production, but the same amount of Br^- had no effect in the absence of Cl^-.[53] This result provided additional evidence for enzymatic formation of a diffusible oxidized chlorine species; this species could oxidize Br^- efficiently at Br^- concentrations which, in the absence of Cl^-, were too low to support direct enzymatic formation of HOBr or Br_2. Thus, the *solution* reaction of enzymatically generated HOCl with Br^- produces Br_2, which then oxidizes H_2O_2. In effect, the Br^-/Br_2 couple has the proper redox potential to function effectively as a "catalyst" of H_2O_2 decomposition by diffusible oxidized chlorine species produced by chloroperoxidase. This effect of Br^- protects the enzyme from autocatalyzed destruction by the reaction products. The "suicide" reaction of chloroperoxidase is a well-documented phenomenon,[9,10,53] which can account for the observed inhibition of the catalatic reaction by moderate, but not high,

levels of the ultra-pure (low-Br^- content) Cl^-. The study cited illustrates that the composition of the reaction mixture, especially the relative concentrations of redox-active components (added intentionally or inadvertently), influences the fate of the diffusible halogenating species produced by chloroperoxidase. Other haloperoxidases with low intrinsic catalase-like activity have been shown to have a halide-dependent pseudocatalatic activity; all such reactions, like the reaction of HOCl with H_2O_2, produce singlet O_2 in high yield.[99-102] Chloroperoxidase and myeloperoxidase appear to be unique among haloperoxidases in having significant halide-independent true catalatic activities.[11,53,103] However, myeloperoxidase exhibits this activity only at very low H_2O_2 concentrations,[103] and cannot sustain the decomposition of large H_2O_2 concentrations, as chloroperoxidase can. Unlike halide-stimulated H_2O_2 decomposition by chloroperoxidase, the true catalatic activity of the enzyme apparently produces O_2 in a triplet state.[102]

Finally, we describe oxygen transfer reactions of chloroperoxidase, some of which will be considered in more detail in subsequent sections. Defining the mechanisms of these reactions and establishing the source of the oxygen atom in the product have been difficult experimental challenges. The first example of chloroperoxidase-mediated oxygen transfer is, appropriately, the H_2O_2-supported oxygenation of chloride, and probably other halides, as well. Due to the chemical reactivity of HOCl with acceptor substrates, halides, H_2O_2, and also the enzyme responsible for its formation (as discussed above), the experimental evidence for formation of HOCl has been indirect, based primarily on similar reactivities of HOCl and the enzymatic chlorinating system. Additional evidence for formation of free HOCl will be discussed in Section IV.A.1. Related to the halide oxygenation activity of chloroperoxidase are two other activities unique to this enzyme: oxidation of I_2 to IO_3^- with H_2O_2,[104] and oxidation of the radical species chlorine dioxide ($ClO_2^{\cdot}$) to ClO_3^-.[105] It should be noted that both chloroperoxidase and horseradish peroxidase can, but myeloperoxidase cannot,[5] utilize chlorite (ClO_2^-) for chlorination of monochlorodimedone.[106] These reactions apparently involve enzymatic oxidation of ClO_2^- to $ClO_2^{\cdot}$, and subsequent nonenzymatic halogention of the substrate by the reactive $ClO_2^{\cdot}$ free radical.[106,107] The "catalatic" activity of chloroperoxidase toward *m*-chloroperoxybenzoic acid which results in scrambling of oxygen atoms in the O_2 evolved (discussed above) also appears to be an example of an oxygen-transfer reaction.[97]

The action of chloroperoxidase halogenating systems on *p*-chloroaniline has been described.[67] At pH less than 5.0, halogenation of the substrate at the 2 and 2,6 positions was the predominant reaction; at higher pH in the presence or absence of halide, only the nitroso product was formed.[67] These results can be rationalized in terms of characteristic reactivities of chloroperoxidase in different pH ranges: halide oxidation typically occurs only at pH below 5.0,[10,11,52,94] whereas the one-electron oxidation of typical peroxidase substrates occurs at higher pH values.[10,11] The *N*-oxidation of *p*-chloroaniline via the hydroxylamine (which was detected under certain experimental conditions) occurred in a pH range[67] where chloroperoxidase can catalyze dehydrogenation of typical peroxidase substrates but cannot oxidize halides efficiently. The dehydrogenation reactions of anilines by horseradish peroxidase have been well characterized and shown to proceed via the unstable radical cation of the substrate.[108] However, under certain experimental conditions horseradish peroxidase can oxidize aniline to nitrosobenzene.[108] Although the mechanism of this "oxygenation" reaction has apparently not been established, the oxidation of anilines by these enzymes represents another example of a radical reaction for which the product distribution depends sensitively upon experimental conditions. From the published data, it cannot be decided whether the chloroperoxidase-catalyzed *N*-oxidation of *p*-chloroaniline represents a general peroxidase-initiated autoxidation of the substrate by molecular O_2, or direct transfer of an oxygen atom from H_2O_2 mediated by the enzyme.

There are several examples of oxygenation on sulfur catalyzed by chloroperoxidase. Conversion of dimethylsulfoxide to the sulfone, in the absence of halide, is a reaction catalyzed

by chloroperoxidase but not other haloperoxidases.[109] However, chloroperoxidase appears not to cataluze a similar reaction with other sulfoxides, such as methionine sulfoxide.[69] On the other hand, several haloperoxidases can oxidize both disulfides and dialkylsulfides to the respective sulfoxides, in reactions requiring halide anion.[73] Specific examples of halide-independent arylalkylsulfide oxidation reactions of chloroperoxidase and horseradish peroxidase will be covered more thoroughly Section IV.B.2. The pseudohalide thiocyanate (SCN^-) can be oxidized to HOSCN by most haloperoxidases.[110] Finally, chloroperoxidase-catalyzed oxygenation of styrene and a few less reactive alkenes to the corresponding epoxides, in the presence of high concentrations of H_2O_2, has been reported.[17,18] In a series of elegant studies undertaken to elucidate reaction mechanisms of cytochromes P-450, Ortiz de Montellano and colleagues have shown that the chloroperoxidase-catalyzed oxygenation of styrene by H_2O_2 produces styrene oxide with stereochemistry preserved, analogous to the O_2-dependent reaction catalyzed by cytochrome P-450.[18] As the best documented oxygen transfer reaction catalyzed by both enzymes, the styrene epoxidation reaction provides a critical test of the degree of correspondence of catalytic function of these two hemeproteins with remarkably similar heme structures. The results of Ortiz de Montellano et al.[18] will be discussed in more detail in Section IV.B.1.

IV. CHLOROPEROXIDASE REACTION MECHANISMS

A. CHLORINATION REACTIONS

1. Evidence for Enzymatic Generation of Free HOCl

The mechanism of chloroperoxidase-catalyzed chlorination reactions has been the subject of considerable interest. Concerning the identity of the enzymatically produced chlorinating species, data have been variously interpreted as supporting one of two possible forms of "active chlorine": free HOCl[2,52,98] and an OCl^- species coordinated to the heme iron (III) of chloroperoxidase.[69,111,112] It is possible that the latter species could be the precursor of the former. However, the two forms might be distinguished only by subtle differences in chemical reactivity related to the site of chlorination of the acceptor molecule, e.g., at the enzyme active site or in solution, by a purely chemical reaction involving free HOCl, or other active chlorine species in equilibrium with HOCl. Neidleman and Geigert[2] have recently summarized the data which support a solution reaction of the halogen acceptor molecule with free HOCl, with no direct participation of the enzyme. The most definitive evidence that chloroperoxidase produces an HOCl-like species from H_2O_2 and Cl^- is the distribution of geometric and chiral isomers among the products of certain reactants. For example, the alpha, beta-halohydrin products of enzymatic chlorination or bromination of many alkenes are racemic. Some of the alkenes examined include *cis*- and *trans*-propenylphosphonic acid,[113] propylene, styrene, *trans*-cinnamic acid,[114] and certain bicycloheptene structures.[115] In every case, the lack of stereospecificity observed in the products is characteristic of the chemical chlorinating agent HOCl. This result indicates that the chlorination reaction probably occurs in solution, without any direct involvement of the enzyme. Another example of lack of specificity is the chlorination of anisole by HOCl and a chloroperoxidase chlorinating system. Both reactions produced the same mixture of *para*- and *ortho*-monochlorinated products.[116] Also, the oxidation of methionine by a chloroperoxidase-H_2O_2-Cl^- system produced a 50:50 diastereomeric mixture of methionine sulfoxide,[115] which suggests that a specific interaction of the enzyme with methionine does not occur.

There is a substantial amount of indirect evidence for formation of free HOCl in chlorination reactions mediated by both myeloperoxidase and chloroperoxidase. Myeloperoxidase has been shown to produce a mixture of HOCl and Cl_2 from H_2O_2 and Cl^- in the absence of a chlorine acceptor molecule.[5] Because these oxidized chlorine species react readily with both the enzyme and H_2O_2, a flow system, with myeloperoxidase immobilized to reduce its contact time with the reaction products, was employed to characterize the reaction.[5] Significantly, addition of *o*-tolidine to trap the oxidized chlorine species did not increase the turnover, i.e., catalytic cycling,

rate of the enzyme. This indicated that the reaction of *o*-tolidine with an "active" enzyme chlorinating reaction was either not rate limiting or did not occur.[5] Chloroperoxidase requires a more acidic pH than myeloperoxidase for oxidation of Cl^-.[4,94] Also, in the absence of a suitable halogen acceptor or reductant, chloroperoxidase is inactivated by the oxidized chlorine reaction products more rapidly than myeloperoxidase.[10] The reason for the differing susceptibilities of the two enzymes to autocatalyzed destruction is not clear; it may relate to the 100-fold greater catalytic efficiency of chloroperoxidase relative to myeloperoxidase.[65,117] Also, the concentration of Cl_2 in equilibrium with HOCl will be greater under more acidic conditions.[118] Although the actual concentration of Cl_2 achieved will be quite small, it may be sufficiently large to rapidly destroy low concentrations of the very active catalyst. One attempt to identify the products of Cl^- oxidation by chloroperoxidase has been reported:[69] bubbling N_2 through a mixture of chloroperoxidase, H_2O_2, and Cl^- at pH 2.8 into NaOH trapped a species with an absorbance spectrum identical to that of Cl_2 trapped in NaOH.[69] It should be noted that oxidation of Br^- by H_2O_2 catalyzed by both chloroperoxidase and horseradish peroxidase yields as the first product either Br_2 or the highly unstable HOBr; in any case, the product reacts with excess Br^- to produce the intensely UV-absorbing Br_3^-.[10,118] The difficulty of directly detecting free oxidized chlorine species produced by chloroperoxidase apparently relates to: (1) the high catalytic efficiency and rather extreme conditions for optimal activity of chloroperoxidase; (2) the pH dependence of equilibria involving Cl^-, HOCl, Cl_2, and Cl_3^- (the latter species exists only at very low pH); and (3) differences in chemical reactivity among corresponding oxidized halogen species generated from different halides. If free HOCl is produced by chloroperoxidase, and if the chlorine acceptor molecule reacts directly with HOCl, or with oxidized chlorine species in equilibrium with HOCl, there is clearly no requirement for binding of the acceptor molecule to the enzyme. To the best of our knowledge, no halogen acceptor substrate has been demonstrated to bind to chloroperoxidase, which would be expected to alter the heme environment and consequently some physical properties of the enzyme. The enzymatic halogenation of MCD shows virtually no dependence on even low concentrations of this chlorine acceptor.[10,111] Also, no significant binding of MCD to chloroperoxidase could be demonstrated by equilibrium dialysis experiments.[10]

This evidence, together with findings presented in Section III, such as the stimulation of chloroperoxidase catalatic activity by Br^- and Cl^-, builds a strong case for HOCl as the elusive, reactive chlorinating species generated from H_2O_2 and Cl^- by action of chloroperoxidase. What is the most probable mechanism of catalysis of Cl^- oxidation by chloroperoxidase? In our attempt to answer this question, we shall first compare published data on the catalytic cycles of chloroperoxidase and other haloperoxidases.

2. Catalytic Cycle of Horseradish Peroxidase

The well-characterized redox states of horseradish peroxidase have served as useful models for probing the possible redox transitions of other hemeproteins during catalysis. The peroxidase isozymes from horseradish root are ferriprotoporphyrin IX-containing glycoproteins which catalyze efficiently the H_2O_2-dependent dehydrogenation of structurally diverse substrates, such as phenols and aromatic amines.[83,84,108] Electron donor substrates of this enzyme typically have a very favorable redox potential for one-electron oxidation. The free radical species resulting from enzymatic oxidation of these compounds have been detected, in many cases, by electron paramagnetic resonance spectroscopy, either directly or by spin-trapping techniques.[84] The final products in these reactions are consistent with the characteristic chemical reactivities of radical intermediates arising from the various electron donors. Since the reactivity of free radical species depends sensitively upon experimental conditions, such as pH and the presence of O_2 and other components in the system, the product profiles and yields resulting from oxidation of a given substrate by a horseradish peroxidase-H_2O_2 system can vary.

Three distinct species of horseradish peroxidase (HRP) are adequate to account for the

catalytic functioning of this enzyme in most reactions. The catalytic cycle, Equation 13, involves a two-electron oxidation of the ferric enzyme by H_2O_2 (and certain other peroxidic agents) to a form designated as compound I.[83,84,108] Two sequential one-electron transfers to the catalyst, involving the intermediate compound II species, return the enzyme to the resting ferric form.

$$\text{HRP - Fe(III)} + H_2O_2 \longrightarrow \text{compound I} + H_2O \tag{13a}$$

$$\text{compound I} + AH_2 \longrightarrow \text{compound II} + AH^{\cdot} \tag{13b}$$

$$\text{compound II} + AH_2 \longrightarrow \text{HRP} - \text{Fe(III)} + AH^{\cdot} + H_2O \tag{13c}$$

$$AH^{\cdot} + AH^{\cdot} \longrightarrow AH - AH \left(\text{or } AH_2 + A_{Ox}\right) \tag{13d}$$

Ferric HRP, compound I, and compound II have distinctly different absorbance spectra,[108] which has facilitated studies of the enzyme under single turnover conditions, that is, with sequential addition of near-stoichiometric amounts of oxidant and reducing substrates. Compound I is rapidly reduced to compound II by exogenous electron donors AH_2. In their absence, compound I has limited stability and, after some minutes, decays spontaneously to compound II, due most likely to transfer of an electron from appropriate amino acid residue(s) of the protein.[119] Transfer of an electron to compound II, typically the rate-limiting step of the catalytic cycle, regenerates the Fe(III) form of HRP.[119] Proton transfer during the catalytic cycle is more difficult to monitor. One H_2O_2 molecule is ultimately reduced to two molecules of H_2O, one of which is probably associated with the heme group as the sixth ligand at the end of the cycle, and can be displaced by other ligands, such as CN^-, or by H_2O_2 to initiate catalysis.[120] Detailed studies of compound I of HRP have established the electronic state of the heme group: one oxidizing equivalent is associated with the iron atom as an Fe(IV)-O (ferryl) species and the second, with a pi-cation radical species of the porphyrin ring.[121] Because the electronic structures of the iron atoms of compound I and compound II have been shown to be very similar, the effective oxidation state of iron in compound II is also considered to be +4.[119,121] Thus, the electron supplied to compound I enters the orbital of and pairs with the unpaired electron on the porphyrin ring. That compound I is not an enzyme-substrate complex has been demonstrated by formation of this species by reaction of ferric HRP with various peroxidic and other one- and two-electron oxidants, the reduction products of which are released into solution.[56]

The reversibility, standard half-cell reduction potentials, and rate constants of the one-electron transfer steps of Equation 13 were established in a careful, thorough study by Hayashi and Yamazaki.[122] Below pH 6.5, the standard half-cell potentials for one-electron reduction of compound I and compound II of HRP isozyme A_2 were nearly identical, with a value near 1 V at pH 6.0.[122] The standard half-cell potential for compound II (but not compound I) reduction showed a marked decrease with increasing pH.[122] Since this pH dependence was characteristic of the particular isozyme used, it was attributed to the ionization of a heme-linked group, with a characteristic pK_a value for each isozyme examined.[122] For both compound I and compound II, the standard half-cell potential and log k, the rate constant for one-electron reduction showed parallel pH dependences.[122] However, in the acidic pH range, the rate of compound I reduction was about 10 times greater than compound II reduction.[122] Thus, the greater instability of compound I, compared to compound II, is related to kinetic rather than thermodynamic properties, since the two species have nearly identical redox potentials in this pH range. The explanation offered for the marked difference in reactivity of the two higher oxidation states of horseradish peroxidase with electron donors was a relatively greater stabilization of the electron transferred to compound I, i.e., by delocalization over the porphyrin ring, compared to the highly localized nature of the electron transferred to the iron atom of compound II.[122]

The ability of horseradish peroxidase to function as an iodoperoxidase can be accommodated

by a modification of the catalytic cycle, Equation 13 in which I^- functions as electron donor.[119] However, detailed kinetic studies have failed to reveal any evidence for involvement of compound II in I^- oxidation.[123] This indicates either that I^- undergoes a concerted two-electron oxidation by compound I or that compound II, if formed, is reduced faster than it is generated.[123] The first detectable product of I^- oxidation is I_2, which can iodinate substrates such as tyrosine or, in the presence of excess I^-, can form I_3^-,[123] analogous to Br_3^- formation. However, recent data have suggested that the first product of I^- oxidation by lactoperoxidase and thyroid peroxidase may be the unstable OI^- species, which is released free in solution.[58] The evidence for OI^- formation was a demonstration of I^- catalysis of the low rate of H_2O_2 decomposition by both iodoperoxidases.[58] This effect is quite similar to halide stimulation of chloroperoxidase catalatic activity and Br^- stimulation of HRP catalatic activity (Section III.D). It was proposed that the oxidized species responsible for H_2O_2 oxidation was OI^-.[58] Other evidence suggested that OI^- might be the species active in iodination of organic substrates.[58] A possible route of formation of HOI was proposed, which accounted for the observation that with lactoperoxidase OH^- was formed faster than substrate was iodinated:[58]

$$E\text{-}Fe(III) + H_2O_2 \longrightarrow E - Fe(V) - O + H_2O \quad (14a)$$

$$E - Fe(V) - O + I^- \longrightarrow E - Fe(III) - OI^- \quad (14b)$$

$$E - Fe(III) - OI^- + H_2O \longrightarrow E - Fe(III) + HOI + OH^- \quad (14c)$$

In this scheme, the net oxidation state of compound I of lactoperoxidase (E) is indicated only as +5. The two-electron oxidation of I^- is considered to be an oxygenation of the substrate, that is, formation of an O-I bond rather than discrete transfer of single electrons from I^- to compound I. Thus, the ferric enzyme would be regenerated directly without the involvement of compound II, consistent with data obtained under single turnover conditions.[58] In order to explain why OH^- was formed more rapidly than substrate was iodinated, it was proposed that the E-Fe(III)-OI^- species was hydrolyzed to produce free HOI, which had a finite existence in solution before reacting with H_2O_2 or iodine acceptor substrate.[58] However, release and subsequent protonation of the OI^- species is a reasonable alternative to Equation 14c. The postulated E-Fe(III)-OI^- intermediate in this scheme must be very unstable, since it has not been detected for any iodoperoxidase by rapid-mixing spectroscopic techniques with dead times in the msec range.[123]

The question that arises is whether bromination and chlorination reactions catalyzed by haloperoxidases involve enzymatically generated OX^-, by analogy to iodination reactions. It has been shown that both chloroperoxidase and HRP can catalyze oxidation of Br^- by H_2O_2 to Br_2.[10,52] With both enzymes, in the presence of limiting H_2O_2, excess Br^-, and absence of bromine acceptor, Br_2 is readily converted to Br_3^-, detected by its intense UV absorbance.[10,52] At larger H_2O_2 concentrations, which sustain Br_2 formation faster than it can be trapped by Br^-, H_2O_2 is oxidized by the oxidized bromine species, accounting for Br^- stimulation of the catalatic activities of both enzymes (Section III.D).[11,53] Many bromination reactions of chloroperoxidase have been characterized,[2,10,52] but early attempts to demonstrate substrate bromination by HRP were actually negative, because this enzyme is a very poor catalyst of Br^- oxidation compared to chloroperoxidase, and optimization of reaction variables is critical. However, at least two examples of bromination reactions which can be catalyzed by either HRP or chloroperoxidase have been reported, the bromination of antipyrine[52] and of MCD, under proper experimental conditions.[98] Compared to the analogous chloroperoxidase reaction, Br^- oxidation catalyzed by HRP requires higher concentrations of both halide and enzyme to force formation of various products possible under different reaction conditions.[98] Moreover, bromination of MCD by the HRP system, but not the chloroperoxidase system, is extremely sensitive to the presence of O_2 (Section IV.A.4.d). Since Br_2 has been identified as a product of Br^- oxidation by both

hemeproteins, and since a chemical bromination system with Br_2 yields the same products as the enzymatic bromination reactions, it has been concluded that Br_2 is the actual brominating agent produced by both enzymes.[10,52,69] However, distinguishing HOBr and Br_2 as the chemical brominating species in these systems would be very difficult. It is possible that OBr^- is the species released from the enzyme and that HOBr may be responsible for at least some reactions attributed to Br_2.

3. Does Chloroperoxidase compound I Participate in Cl^- Oxygenation?

The formation of compound I of chloroperoxidase with several peroxidic agents, including H_2O_2, ethyl hydroperoxide, and peracetic acid, has been characterized.[124] As with other true hemeprotein peroxidases, the absorbance spectrum of compound I does not appear to depend upon the identity of the oxidant, indicating that a moiety of the organic peroxide does not remain closely associated with the oxidized heme group.[124] The absorbance spectrum of compound I of chloroperoxidase has the characteristic low-intensity broad Soret band of other compound I species, shifted, however, to 367 nm compared to 400 nm for HRP.[108,125] The absorbance bands of compound I species in the visible region are quite characteristic of each hemeprotein, i.e., at 610 nm (weak) and 698 nm for chloroperoxidase,[125] and broad bands at 577, 622, and 651 nm for HRP compound I.[108] Compound I of chloroperoxidase formed with H_2O_2 is quite unstable, in part because of the large intrinsic catalatic activity of the enzyme (Section III.D), and, thus, has been very difficult to characterize under single-turnover conditions approximating those optimal for catalysis.[124] Accurate spectral and kinetic data on the formation of chloroperoxidase compound I have been obtained by use of peracetic acid, which will support at least some chlorination reactions of chloroperoxidase.[111] It has been assumed but not definitely established that enzymatic chlorination reactions with H_2O_2 and peracetic acid proceed by the same mechanism.

Since definitive data on the existence of a short-lived Fe(III)-OX^- intermediate of chloroperoxidase or any other haloperoxidase are lacking, we consider other data which might indicate that the mechanism of chloroperoxidase catalysis of Cl^- oxidation is similar to that proposed for I^- oxidation by HRP (Equation 14). The rapid conversion of compound I of HRP to the ferric enzyme by reaction with I^- provides strong evidence that compound I is a requisite intermediate in I^- oxidation.[123] Does Cl^- similarly accelerate the decomposition of chloroperoxidase compound I? The limited published data relating to this question are not entirely consistent. For example, Thomas first reported that Cl^- and Br^- have quite different reactivities with chloroperoxidase compound I.[10] By use of stopped-flow spectrophotometry, Thomas showed that rapid mixing of compound I of chloroperoxidase with Br^-, at threefold molar excess with respect to compound I, greatly accelerated the decay of the enzyme intermediate, but a 300-fold molar excess of Cl^- had little effect on compound I stability.[10] More recently, another laboratory reported that Cl^-, at four times the concentration used by Thomas,[10] greatly accelerated the decay of chloroperoxidase compound I.[112] It would not appear that the higher Cl^- concentration used in the study of Dunford et al.[112] could explain this significant difference in compound I reactivity, since the large excess of Cl^- used by Thomas[10] should have been adequate to reduce an enzyme intermediate with reduction potential sufficiently positive to oxidize Cl^-.

The rapid decay of chloroperoxidase compound I induced by near-stoichiometric amounts of Br^- is consistent with other data, such as halide stimulation of catalatic activity of the enzyme (Section III.D),[11,53] which demonstrate that the enzyme oxidizes Br^- more efficiently than Cl^-. This property of the enzyme suggests one explanation for the apparent discrepancy between these reports of compound I reactivity with Cl^-: different Br^- contamination of the NaCl used by the two labs. The study of halide stimulation of chloroperoxidase catalatic decomposition[53] identified variability among AR grade chloride salts from different sources which was clearly attributed to variable Br^- contamination. For certain chloride salts, Br^- content was sufficiently large to alter the observed effects of Cl^- in the millimole per liter of concentration range

employed in the studies of both Thomas[10] and Dunford et al.[112] Since Cl^- contaminated by Br^- would, at high concentrations, increase the rate of decay of chloroperoxidase compound I,[10] the original report of Thomas[10] must be considered to be more accurate, i.e., that compound I is quite unreactive with Cl^-. One additional comment should be made about the study of Dunford et al.:[112] the authors acknowledged that the citrate buffer employed appeared to serve as a halogen acceptor substrate in experiments with both the chloroperoxidase and HOCl (control) chlorinating systems, but possible effects of this side reaction on the kinetics of compound I decay observed in the *presence* of Cl^- were not considered.[112] Thus, it has not been convincingly demonstrated that Cl^- can accelerate the decay of chloroperoxidase compound I at catalytically significant rates, especially since the earlier data of Thomas[10] indicated that Cl^- does not have this effect. Although these single-turnover experiments are difficult to perform and consume large amounts of enzyme, the important question of Cl^- acceleration of chloroperoxidase compound I decay needs to be resolved by a systematic investigation, with careful control of the experimental variables.

Since a specific interaction of Cl^- with chloroperoxidase is a critical aspect of enzymatic Cl^- oxidation, it is appropriate to review the published data relating to the effects of Cl^- on various physical and catalytic properties of chloroperoxidase. Thomas et al. first reported that high concentrations of both Cl^- and Br^- altered the absorbance spectrum of ferric chloroperoxidase by shifting the Soret absorbance, near 400 nm, to about 420 nm.[10] In a very careful, thorough study of ligand binding to the enzyme, Sono et al.[126] reported that Cl^- produced a unique change in the spectrum of the ferric enzyme not observed with any other halide: the Soret absorbance maximum was shifted from 399 to 422 nm, quite consistent with Thomas' result.[10] However, both Br^- and I^- shifted the Soret absorbance to somewhat shorter wavelengths, near 393 nm. The spectrum of the F^- complex of ferric chloroperoxidase, with a Soret absorbance centered at 409 nm, was different from those of the other halide complexes.[126]

The apparent dissociation constants of the Br^- and Cl^- complexes of ferric chloroperoxidase are comparable and show similar pH dependences, increasing from a value of 20 to 30 mmol/l at pH 2.0 to 400 to 660 mmol/l at pH 3.5.[15] This pH dependence was attributed to binding of the halide anion to a protonated species ($pK_a < 2.0$) of the enzyme.[15] It is of interest that high concentrations of Cl^- convert the ferric enzyme from a high-spin ($S = 5/2$) to a low-spin ($S = 1/2$) state at room temperature,[3,15] whereas substrate binding to ferric cytochrome P-450, which occurs at near-stoichiometric substrate concentrations in the case of cytochrome $P\text{-}450_{cam}$, converts this enzyme from a low- to high-spin form.[28,50] Conclusive identification of the binding site of Cl^-, presumably as the sixth ligand of the heme iron, by low-temperature spectroscopic techniques that probe the iron environment has not been accomplished, because the native, halide-free enzyme undergoes spin-state transitions as a function of both temperature and pH.[3,15] At a constant Cl^- concentration of 20 mmol/l, the extent of formation of the low-spin Cl^- complex of ferric chloroperoxidase at room temperature depends on pH in a manner very similar to the MCD chlorination activity of the enzyme; both parameters decrease smoothly from a maximal value near pH 2.8 to almost zero at pH 5.0.[94] The interpretation of these results was that Cl^- binding to the ferric enzyme is prerequisite for catalysis of Cl^- oxidation.[94]

More detailed studies have demonstrated that the effects of Cl^- on the chlorination activity of chloroperoxidase are quite complicated. For example, the pH optimum for MCD chlorination depends on Cl^- concentration: as Cl^- is increased, the pH optimum shifts to higher pH and the turnover number increases by a factor of three to four.[94] Consequently, at high Cl^- concentrations, there is relatively less activity at the lower pH values where a greater fraction of the ferric enzyme exists as the Cl^- complex.[15] A steady-state kinetic analysis of the enzymatic chlorination of MCD with peracetic acid suggested an explanation for this effect: Cl^- binds to a protonated species of the ferric enzyme, whereas peroxides react with an unprotonated species.[111] Thus, Cl binding inhibits compound I formation by inhibiting the rate of equilibration of the protonated ferric species with the unprotonated peroxide-reactive form. This indicates that the protonated

Cl^- complex of ferric chloroperoxidase cannot be directly converted to compound I by peroxides. The effects of Cl^- on myeloperoxidase catalytic properties have been reported to be very similar[126,127] to those observed with chloroperoxidase. Since Cl^- functions as inhibitor and substrate in both enzymatic chlorinating systems, a more detailed analysis of the effects of Cl^- on myeloperoxidase activity was undertaken.[126] The results suggested the presence of two Cl^- binding sites on the enzyme: a pH-dependent (protonated) inhibitor site, and a pH-independent substrate site.[127] For myeloperoxidase, the evidence for binding of Cl^- at the sixth ligand position of the heme iron is more convincing;[128] this can account for Cl^- inhibition of reaction of the protein with H_2O_2.

These enzymatic chlorinating systems are complicated further by effects of Cl^- on chemical equilibria and nonenzymatic reactions, which may influence the overall reaction kinetics. For example, the Cl^- concentration will influence the solution equilibrium involving Cl^-, H^+, enzymatically produced HOCl, and Cl_2.[118] Although the steady-state Cl_2 concentration in these reactions will never be very large even under the most acidic conditions that the enzymes will tolerate, Cl_2 is more reactive than HOCl.[118] Thus, the pH dependence of Cl_2 formation would be expected to influence rates of chlorination and oxidation of compounds in these systems. The stimulatory effect of added Cl^- or Br^- on the oxidation of H_2O_2 by HOCl has been shown to depend on pH in a manner consistent with involvement of Cl_2 or Br_2.[53] Moreover, Cl^- catalysis of MCD chlorination by HOCl has been reported,[112] which can be similarly attributed to Cl_2 involvement in the reaction. Since both HOCl and Cl_2 can inactivate chloroperoxidase and myeloperoxidase,[5,10] the rates of inactivation should depend on the rates of enzymatic generation of oxidized chlorine species: as the pH optimum is approached from the more alkaline region, faster rates of Cl^- oxidation should lead to more rapid enzyme inactivation and to more pronounced inhibition of the overall rate. Under more acidic conditions, significant binding of Cl^- by these enzymes may serve a dual function in preventing their autocatalytic destruction by: (1) inhibiting the reaction of H_2O_2 with the ferric enzymes, thereby inhibiting the rate of generation of HOCl; and (2) similarly inhibiting the reaction of oxidized chlorine species with the enzymes. There is published evidence for such an inhibitory effect of Cl^- on myeloperoxidase self-inactivation.[127] Collectively, these results suggest that Cl^- may play several important roles in modulating the rate of its oxidation by chloroperoxidase and myeloperoxidase.

High concentrations of HOCl added to hemeproteins rapidly destroy the heme group.[9,129] HOCl is also thought to play a role in the autocatalytic destruction of chloroperoxidase and myeloperoxidase during Cl^- oxidation in the absence of a halogen acceptor or reductant.[9,10] However, Cl_2, in equilibrium with HOCl and a very high Cl^- concentration, may be the species primarily responsible for heme destruction in these reactions. The reactions of HOCl with various hemeproteins under single-turnover conditions in the absence of Cl^- have also been described. Chance first reported that reaction of horseradish peroxidase with a 20-fold excess of HOCl at pH 5.4 and 8°C resulted in rapid formation of compound I, which decayed slowly to compound II.[130] These spectral changes were similar to those produced with stoichiometric amounts of H_2O_2 under the same conditions.[130] The calculated pseudo-dissociation constant for HOCl was about 100 times larger than that for H_2O_2, suggesting a strong preference of the enzyme for H_2O_2 instead of HOCl.[130] It was noted that the data could not eliminate the possibility that HOCl produced H_2O_2, which might be responsible for compound I formation.[130] Hollenberg et al. also described studies of the reaction of HRP with NaOCl carried out in citrate-phosphate buffer, which may have influenced their results (*vide supra*).[106] They were unable to titrate the ferric enzyme with low concentrations of NaOCl; the predominant species formed was compound II, but other unidentified heme species were apparently produced, since the ferric form could not be fully regenerated by adding reductants subsequent to HOCl.[106] The nonenzymatic reaction of excess HOCl with reductants added to generate compound II from compound I complicated the data and interpretation.

Harrison and Schultz investigated the reaction of myeloperoxidase with HOCl.[5] At pH 8.6

in the absence of Cl^-, reaction of myeloperoxidase with a 10-fold excess of either H_2O_2 or HOCl produced approximately the same amount of compound II.[5] When the same experiment was conducted at pH 4.5, similar results were obtained, with H_2O_2 producing relatively more compound II and HOCl somewhat less, than at pH 8.6.[5] Compound I was shown by stopped-flow spectrophotometry to be the first, very transient intermediate formed by reaction of myeloperoxidase with H_2O_2 or HOCl.[5] When 50 mmol/l Cl^- was included in each of these reactions at pH 4.5, no observable changes in the spectrum of myeloperoxidase occurred, even though the formation of oxidized chlorine was demonstrated in the H_2O_2 system.[126] The interpretation of these results presented a dilemma, since they suggested that oxygen atom transfer between Cl^- and compound I, presumed to be responsible for Cl^- oxidation, was reversible. If HOCl-dependent conversion of the enzyme to compound I occurred to any significant extent, then enzymatic dehydrogenation of halogen acceptors such as MCD, an excellent one-electron reductant for peroxidases (Section IV.A.1.d), would be very competitive with chlorination. However, under normal steady-state conditions of chlorination, the relative concentrations of the reactants, i.e., enzyme = HOCl << halogen acceptor, will ensure that dissociation of HOCl from the enzyme and subsequent rapid reaction of HOCl (or Cl_2) with the halogen acceptor will be very probable. Thus, both kinetic and thermodynamic properties of these reactions should preclude the accumulation of HOCl to concentrations that would allow it (or Cl_2) to react with the enzyme, at least during the steady-state of chlorination. However, after the acceptor has been depleted, accumulation of HOCl, which could result in either reversal of compound I formation and/or destruction of the heme group, would be a very effective means of terminating the reaction.

The fact that HOCl has been shown to react with the heme group of peroxidases under single-turnover conditions, but apparently does not do so under steady-state conditions of chlorination, emphasizes the difficulty of defining the detailed reaction sequence of these extremely complicated reactions. However, such results provide indirect support for multiple roles of Cl^- in these reactions. Thus, Cl^- should inhibit HOCl-dependent compound I formation more effectively than it inhibits reaction of H_2O_2 with the ferric enzymes because of the lower affinity of HOCl, compared to H_2O_2, for the hemeproteins. Also, the function of Cl^- in "promoting" the formation of Cl_2 from HOCl may serve to protect the enzyme, as long as halogen acceptor is present: Cl_2 will be produced in very small concentrations in solution, rather than at the enzyme active site. This will increase the probability of reaction of Cl_2 with the large excess of halogen acceptor, which will effectively maintain Cl_2 concentrations very low, until the acceptor has been consumed. The fact that HOCl-induced changes in the absorbance spectrum of myeloperoxidase observed in the absence of halide were prevented by a large Cl^- concentration is consistent with Cl^- inhibition of reaction of HOCl with the enzyme.[5] The probability of reaction of enzymatically generated HOCl with myeloperoxidase or chloroperoxidase during the *steady-state* of chlorination cannot be readily determined, but is presumably very low until the halogen acceptor concentration has been depleted.

The inherent instability of compound I species of both myeloperoxidase and chloroperoxidase, even in the absence of Cl^-, is unlike higher oxidation states of many other hemeproteins, known to be directly involved in product formation. There is experimental evidence that chloroperoxidase compound I participates in the catalatic activity of the enzyme.[124] However, H_2O_2 decomposition by this enzyme appears to be a dehydrogenation reaction, analogous to the catalase-mediated reaction, and not an oxygen atom transfer reaction. The published evidence does not establish convincingly any direct role for compound I species in Cl^- oxidation by chloroperoxidase.

4. Proposal for Chloroperoxidase Compound III as the Cl^- Oxygenating Agent

a. General Aspects of the Structure and Reactivity of Compound III Species

The difficulty of establishing experimentally that chloroperoxidase compound I has an

essential role in Cl^- oxidation is most likely related to thermodynamic constraints on the discrete reactions of the catalytic cycle, which are imposed by the thermodynamics of the overall reaction:

$$H_2O_2 + 2H^+ + 2e^- \longrightarrow 2\ H_2O \qquad E^\circ = 1.77\ V \qquad (15a)$$

$$2\ Cl^- \longrightarrow Cl_2 + 2e^- \qquad E^\circ = -1.36\ V \qquad (15b)$$

$$H_2O_2 + 2\ Cl^- + 2\ H^+ \longrightarrow Cl_2 + 2\ H_2O \qquad E^\circ = 0.41\ V \qquad (15c)$$

Under standard conditions (when the activity of all components is 1), E° for the overall reaction is +0.41 V, $\Delta G^\circ = -n\ F\ E^\circ$ is negative, and product formation is thermodynamically favorable. The half-cell reduction potential for H_2O_2 depends on pH, decreasing by 0.06 V for each unit increase in pH. Therefore, at pH 5.0, where E° for the overall reaction is 0.11 V, oxidation of Cl^- by H_2O_2 is less favorable. However, the effect of decreasing the H^+ concentration on E° can be compensated by increasing the H_2O_2 concentration and/or decreasing the Cl_2 concentration; reaction of Cl_2 with a halogen acceptor molecule or with H_2O_2, while either is available, will effectively maintain the Cl_2 concentration very low.

For HRP, it can be easily demonstrated that *each* reaction in the catalytic cycle of halide oxidation by H_2O_2 is also thermodynamically favorable. Since thermodynamic parameters are independent of the reaction pathway, and E° values are related to thermodynamic parameters, the formation of HRP compound I can be considered to proceed reversibly by loss of single electrons via compound II. Thus, ΔG° for two-electron reduction of compound I will be the same whether the two electrons are transferred singly or as a pair, and ΔG° for two-electron oxidation of the ferric enzyme will have the same magnitude and opposite sign.[122] In the reaction sequence below, E° of 1.0 V is appropriate for the *two-electron* reduction of compound I (Equation 16c), since it is the average of the standard one-electron reduction potentials of compound I and compound II of HRP, which have nearly identical E° values of 1.0 V at pH 5.0.[122] Thus, the net ΔG° for the two-electron conversion of compound I to the ferric enzyme will be $-2\ n\ F$, with $-n\ F$ contributed by each one-electron reduction step. Since all half-reactions are written as two-electron tranfer reactions, ΔG° for each half-reaction, and for the overall reaction, is strictly proportional to the respective E°. At pH 5.0, the enzymatic oxidation of Br^- by H_2O_2 can be written as the sum of four half-reactions, with standard redox potentials as indicated:

$$E\text{ - Fe(III)} \longrightarrow \text{compound I} + 2\ e^- \qquad E^\circ = -1.0\ V \qquad (16a)$$

$$H_2O_2 + 2\ e^- + 2\ H^+ \longrightarrow 2\ H_2O \qquad E^\circ = 1.47\ V \qquad (16b)$$

$$\text{compound I} + 2\ e^- \longrightarrow E - Fe(III) \qquad E^\circ = 1.0\ V \qquad (16c)$$

$$2\ Br^- \longrightarrow Br_2 + 2e^- \qquad E^\circ = -1.07\ V \qquad (16d)$$

$$H_2O_2 + 2\ Br^- + 2H^+ \longrightarrow Br_2 + 2\ H_2O \qquad E^\circ = +0.40\ V \qquad (16e)$$

The oxidation of ferric HRP by H_2O_2, with E° of +0.47 V, proceeds readily at stoichiometric concentrations of enzyme and H_2O_2 under conditions which are far removed from standard conditions, as has been well documented. For the reduction of compound I by Br^-, E° is near zero; but the experimental E value will be positive, favoring the oxidation of Br^-, if the ratio of Br^- to Br_2 is very large. This has been verified experimentally: the oxidation of Br^- by a HRP-H_2O_2 system absolutely requires a large excess of Br^-[52] and is probably sustained by low Br_2 levels achieved by rapid reaction of Br_2 with a halogen acceptor, with H_2O_2, or with Br^- to produce

Br_3^-.[10,52,53] Although E° for the overall reaction has a value of +0.40, it appears that *each* reaction involving the enzyme must achieve a positive E corresponding to a negative ΔG, in order to drive the reaction in the direction of product formation. A significant fraction of the net ΔG for the complete reaction occurs in the reaction of H_2O_2 with ferric HRP: this energy, derived from reduction of the oxidant, is lost to the environment, since compound I contains only [1.0/1.48] or about 70% of the energy associated with H_2O_2 reduction at pH 5.0. Although the reduction of H_2O_2 at pH 5.0 provides adequate energy to oxidize Cl^-(see Equation 16b and Equation 8), this simple analysis suggests that an oxidized enzyme intermediate with a half-cell reduction potential of 1.0 V could not bring about Cl^- oxidation. Two-electron oxidation of Cl^- would require an oxidized enzyme species with a standard half-cell reduction potential of about 1.4 V, i.e., corresponding to transfer of about 95% of the energy of H_2O_2 reduction to the enzyme at pH 5.0. At lower pH, where the redox potential for H_2O_2 reduction is more positive, the efficiency of energy transfer required would be lower. However, at pH 3.0, which is optimal for Cl^- oxidation by chloroperoxidase, transfer of 88% of the energy of H_2O_2 reduction would be required to produce such a strongly oxidizing compound I.

Because of the thermodynamic constraints on the oxidation of Cl^- by H_2O_2 discussed above and because there is no definitive evidence for involvement of chloroperoxidase compound I in Cl^- oxidation, an alternative hypothesis was proposed in very general terms for the highly "oxidized" enzyme intermediate with E° sufficiently large to oxidize Cl^-.[98] We now elaborate more specific details of this hypothesis. The oxidized enzyme species that oxidizes Cl^- is postulated to contain a bound partially reduced O_2 species, i.e., *both* oxygen atoms of the oxidant with a bond, perhaps "stretched", between the oxygen atoms. Such a species would be expected to have a more positive reduction potential than a compound I species containing only one oxygen atom of the oxidant. Thus, reductive cleavage of a bound O_2 species would be expected to generate more energy than reduction of H_2O_2. The enzyme could facilitate conservation of energy by having Cl^- bound appropriately with respect to the bound O_2 and by providing a reaction path for movement of electrons and atoms such that the energy produced by reductive cleavage of the O-O bond could be transferred into the new O-Cl^- bond. A necessary feature of this hypothesis is considered to be coupling, in space and time, of cleavage and formation of the respective bonds. Close coupling of these events will insure productive transfer of the oxygen atom to the acceptor and reduce the proability that energy is lost to the environment as heat.

Concerning the oxidation state of this hypothetical highly reactive oxygenating chloroperoxidase intermediate, at least two possibilities exist. The first is a ternary complex formed by the binding of first H_2O_2 and then Cl^- to chloroperoxidase: the effective oxidation state of iron in this complex would be more nearly +5 instead of +4, the latter being characteristic of compound I species. The electronic configuration of the cysteinate-ligated heme group of chloroperoxidase might permit at least a fleeting existence to an iron +5 species. The presence of Cl^- bound at the active site of this species would direct its rapid decay to the ferric enzyme and OCl^-, without formation of compound I as a discrete intermediate. In the absence of bound Cl^-, a complex of the ferric enzyme and H_2O_2 might exist as a short-lived precursor to compound I, which, experimental data suggest, cannot oxidize Cl^-. This hypothesis accommodates the experimental observations that Cl^- inhibits chloroperoxidase compound I formation, since compound I would not be produced or have any role in OCl^- formation, according to this hypothesis. The criticisms of this mechanism are twofold: (1) there is no experimental evidence that any of the true hemeprotein peroxidases form an equilibrium, Michaelis-type complex with H_2O_2 prior to compound I formation; indeed, the absence of such a complex appears to be an inherent feature associated with their efficient use of H_2O_2 as an oxidizing substrate; (2) direct use of H_2O_2 for Cl^-oxidation requires that the efficiency of energy transfer from H_2O_2 to O-Cl^- be almost 90%, as discussed above. This is considered a very stringent constraint on energy-coupling phenomena in biological systems. The second possibility for the active oxygenating intermediate of chloroperoxidase is suggested by certain features typical of halogenation

reactions of this enzyme: high concentrations of H_2O_2 are employed and most halogen acceptor molecules have an activated, or easily abstracted, hydrogen atom or electron.[2] Such conditions would be expected to favor formation of chloroperoxidase compound III.

Compounds III of various peroxidases have been produced by several means:[119] (1) by cycling the enzyme with H_2O_2 in the presence of a slight excess of an electron donor such as ascorbate to convert the enzyme to compound II and then adding a large excess of H_2O_2 to produce compound III; (2) by treating the ferrous enzyme with O_2 in the absence of excess reductant; or (3) by reacting the ferric enzyme with O_2. The iron oxidation state and O_2 ligation of compounds III of peroxidases are considered to be generally analogous to dioxygen complexes of ferrous hemeproteins, such as hemoglobin and cytochromes P-450,[36,43] although the absorbance spectra of all such species are not identical. These spectral differences attest to the large number of resonance forms possible for these species. If only the iron center and the bound O_2 are considered, at least three formal resonance forms can be written for compound III, or dioxygen-ferrous, species of hemeproteins: $Fe(II)\cdot O_2$, $Fe(III)\cdot O_2^-$, and $Fe(IV)\cdot O_2^{2-}$. Including the electrons associated with the porphyrin ring, and allowing for differences in protein electronic structure in the vicinity of the heme group of different hemeproteins greatly increases the possibilities for subtle changes in distribution of electrons on the heme-O_2 structure. Additional evidence for differences in electronic distribution among different compound III species has emerged from detailed analysis of resonance Raman spectra.[131] For example, resonance Raman data have revealed that the bound O_2 species of HRP compound III has greater electron density and consequently a weaker O-O bond than O_2 bound to oxy-myoglobin. These results were interpreted to indicate a greater degree of "activation" of the O_2 species of compound III of HRP.[131]

How could compound III react and how do compound III species actually react? The possible reactions of compound III can be visualized most readily by referring to Figure 2, which depicts in very general terms the interrelationships among known oxidation states of hemeproteins; the properties of compounds I and II have already been discussed. Because compound III species of hemeproteins have net oxidation state of +6 and several possible resonance forms, they are capable of more diverse reactions than other oxidation states of hemeproteins. The reactions of compound III species which are, in theory, possible include: (1) dissociation of O_2 from the ferrous resonance form, as occurs with oxy-hemoglobin and oxy-myoglobin; (2) dissociation of O_2^-, yielding ferric heme; (3) one-electron reduction of the ferric-O_2^- resonance form to produce the peroxide species O_2^{2-}, which, upon protonation, could either dissociate as H_2O_2 or convert the ferric hemeprotein to compound I; (4) a net two-electron reduction of the complex that results in transfer of a two-electron deficient oxygen atom to a substrate bound appropriately at the active site, such as a halide anion (in the case of chloroperoxidase) or an organic molecule (in the case of cytochrome P-450). The last-mentioned reaction, which results in a two-electron oxidation of the substrate, might occur spontaneously, but should proceed more rapidly if an electron were supplied to the complex. The spontaneous oxygenation reaction of compound III would produce the poorly active compound II species. However, transfer of an electron to a compound III species poised for oxygen atom transfer to an appropriately situated substrate would serve the functions of: (1) activating O-O bond cleavage, (2) ensuring that the hemeprotein is regenerated as the catalytically competent ferric form, and (3) perhaps most importantly, realizing a higher yield of energy from a net three-electron reduction of a heme-bound O_2 than can be achieved from two-electron reduction of compound III, compound I, or H_2O_2. Although compound III species are in an effective oxidation state of +6, they have been reported to be even less reactive than compound II with typical peroxidase one-electron donor substrates under single turnover conditions.[119] This result probably reflects greater steric and electronic constraints on the reactivity of a dioxygen-heme complex than on other heme oxidation states containing only a single oxygen atom derived from the oxidant, such as compound I. The constraints of activating a bound dioxygen species for oxygen atom insertion into an acceptor

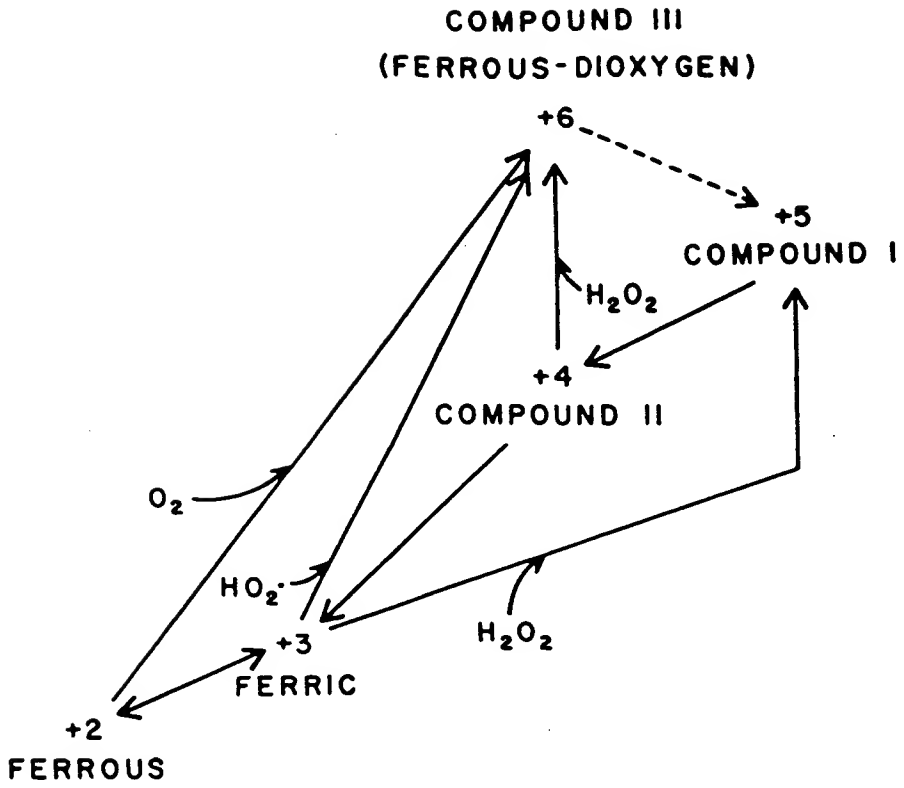


FIGURE 2. Relationships among the possible redox states of hemeproteins. The formal oxidation state of each heme species, which is not strictly equivalent to the iron oxidation state, is indicated by the numbers +2 to +6. The vertical positions of these species correspond roughly to their relative energy content. As shown, net oxidation of selected heme species by two, three, and four electrons can be achieved with H_2O_2, HO_2· (protonated superoxide), and O_2, respectively; however, not all possible reactions are shown, e.g., the poorly understood reaction of H_2O_2 with compound III (+6). In general, two heme species with a difference in oxidation state of +1 can be interconverted by transfer of an electron, at least under well-defined single turnover conditions. However, reactions with H_2O_2 are highly exothermic and irreversible. A broken arrow indicates that the reaction has not been definitely established to occur. This is a general scheme for hemeprotein redox interconversions and cannot depict all details, especially critical proton transfer reactions, which will necessarily vary for individual hemeproteins. This diagram was adapted, with modifications, from Reference 119.

molecule relate to cleavage of the O-O bond and disposition of the energetic oxygen atoms produced. These constraints would be reflected in the reaction surface characteristic of each catalyst leading to the final products.

The complete reduction of O_2 by an enzyme or other catalyst will yield maximal useful energy, i.e., for formation of chemical bonds, only if four reducing equivalents are supplied to the enzyme-O_2 complex within a short time interval. Otherwise, partially reduced O_2 species would likely dissociate from the catalyst, dissipating the energy produced. The direct involvement of a compound III species in an oxygenation reaction would require that one electron be provided by the heme iron, in an effective oxidation state of +2, and that two more electrons be provided by the enzyme-bound oxygen acceptor molecule, oriented properly near the O_2-coordinated heme iron Thus, transfer of an additional electron to the hemeprotein-O_2-substrate complex from another source would satisfy the electron supply criterion optimal for "activation" and reductive cleavage of the bound O_2 and formation of an O-Y bond. However, if the requisite electrons needed for four-electron reduction of the bound O_2 molecule are unavailable, one-electron reduction of the complex may be energetically unfavorable. Indeed, the low reactivity

of compound III species with typical peroxidase one-electron donors may be related to their limited ability to supply multiple electrons and/or accept an O atom, although other factors may also be involved. However, the low reactivity reported for compound III species under single turnover conditions may not accurately reflect their reactivity under dynamic conditions of catalysis, when reactive reducing free radicals, such as O_2^- and radical cations of electron donor substrates and perhaps other radicals, will likely be present.[98,119]

The predominant resonance form of compound III and the constraints of activating this complex will, no doubt, be determined by the immediate protein environment of the heme group, for example, by the presence or absence of a binding site for an oxygen acceptor substrate. Thus, compound III species of different hemeproteins are expected to have quite different reactivities, consistent with published data. However, the efficient, productive "activation" of O_2 for oxygen atom insertion into a substrate bound at the heme active site must dictate certain essential requirements of the active site protein environment, which facilitates precisely timed transfer of the requisite number of electrons, protons, and an oxygen atom in a multicenter reaction.

b. Chloroperoxidase compound III

The properties of chloroperoxidase compound III remained elusive until three laboratories reported independently, within a short time period, the formation of this species by quite different routes.[132-134] Nakajima et al. produced chloroperoxidase compound III at 5°C by reaction of excess H_2O_2 with compound II, which had been generated by cycling the enzyme with limiting H_2O_2 and ascorbate.[132] Sono et al. reduced the ferric enzyme with dithionite under anaerobic conditions at 4°C in a cryogenic buffer, and then generated the ferrous-O_2 complex by cooling this solution to –30°C, and exposing it to O_2.[133] Lambeir and Dunford used rapid-scanning spectrophotometric methods at 25°C to analyze the kinetics of O_2 binding to the ferrous enzyme, produced by anaerobic titration with dithionite.[134] All three groups reported very similar absorbance spectra for chloroperoxidase compound III, i.e., ferrous-O_2 complex, which reinforced previous conclusions about the equivalence of the compound III species and ferrous-O_2 complex formed from a given hemeprotein by different routes.

With absorbance maxima at 354, 430, 554, and 587 nm,[133] the species closely resembled compound III species produced from other peroxidases,[135] but was distinctly different from the ferrous-O_2-substrate complex of cytochrome P-450, with absorbance maxima at 418 and 555 nm.[28] Since the ferrous-CO complexes of both chloroperoxidase and cytochrome P-450 have the characteristic 450-nm absorbance band,[13,28] the marked difference in their ferrous-O_2 complexes was unexpected. Sono et al. characterized the magnetic circular dichroism of chloroperoxidase compound III and also demonstrated that, like analogous species of other hemeproteins, it had no electron paramagnetic resonance signal at 77°K.[133] Moreover, O_2 was easily displaced from the complex by CO, yielding the ferrous-CO complex of the enzyme, in contrast to the behavior of HRP compound III.[133] Comparative data obtained by Sono et al. established the relative rates of decomposition of various compound III species to the respective ferric enzymes: chloroperoxidase compound III was shown to be less stable than HRP compound III or oxymyoglobin by factors of 30 and 300, respectively, under the rather unusual experimental conditions (–10°C and pH 6.0) required for study of the quite reactive chloroperoxidase species.[133] Lambeir and Dunford reported the rate constant for binding of O_2 to ferrous chloroperoxidase as $5.5 \pm 1.0 \times 10^5\ M^{-1}\ s^{-1}$, over the pH range 3.5 to 6.0.[134] A very similar value ($7.7 \times 10^5\ M^{-1}\ s^{-1}$) has been reported for O_2 binding to substrate-bound ferrous cytochrome $P\text{-}450_{cam}$ (from *P. Putida*), at pH 7.4 and 4°C.[136]

Nakajima et al. established that chloroperoxidase compound III, like HRP compound III, has a low catalatic activity. Excess H_2O_2 was decomposed to O_2, concomitant with conversion of compound III to compound II.[132] At pH 4.0, the catalatic activity of compound III was considerably smaller than the catalatic activity of the ferric enyme, but at pH 6.0, H_2O_2 was decomposed by both enzyme species at more comparable rates.[132] The experimental conditions

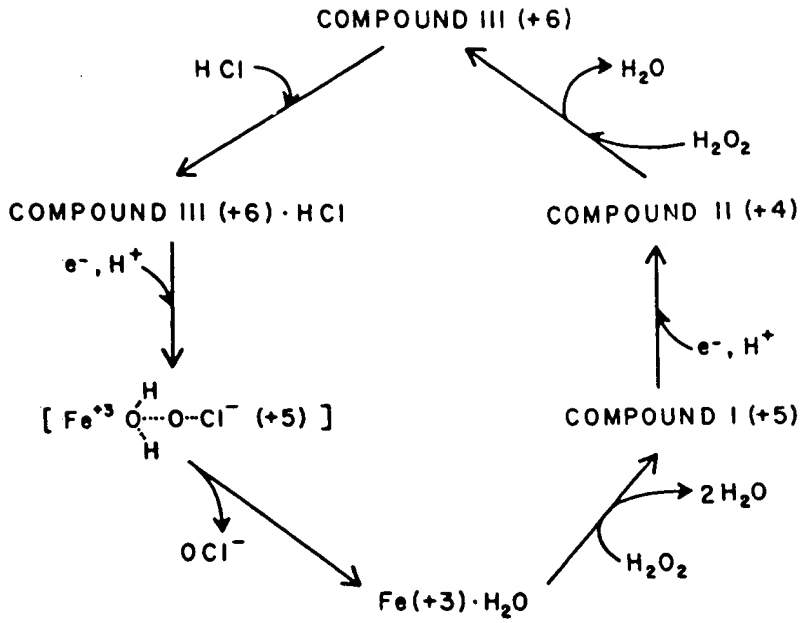


FIGURE 3. Proposed catalytic cycle of chloroperoxidase. The catalytic cycle is depicted as proceeding from the heme species with the lowest energy, the ferric form at the bottom, to compound III, with the highest energy, at the top; however, in this simplified scheme, the relative energies of compounds I and II are actually reversed. Catalysis is initiated by reaction of the resting ferric enzyme with H_2O_2, and proceeds via well-established reactions to compound III. The existence and structures of the two transient species arising from compound III in this cycle are speculative. It is possible that HCl could bind to compound I or II and alter the lifetimes of these species in the cycle. As elaborated in the text, the hemeprotein species that gives rise to OCl^- is postulated to be an unusual complex of heme with a peroxidic species generated *in situ*. This complex (+5 oxidation state) is considered to have a distinct, perhaps weakened, O–O bond and to directly transfer an "activated" oxgyen atom to Cl^- bound at the active site. Although protons are included where they are required for overall charge balance, the points at which they enter the cycle are unknown.

required to record the spectra of chloroperoxidase compound III, together with the data of Sono et al. on the instability of this species,[133] suggest that if it were formed during catalysis of chloride oxidation, it would be very short-lived under typical conditions for these reactions.

c. Proposed Mechanism for Chloroperoxidase Chlorination Reactions

We now consider in greater detail how chloroperoxidase compound III could be directly involved in "oxygenation" of Cl^-. A typical chlorinating reaction mixture contains a catalytic amount of the enzyme, a very high Cl^- concentration, a halogen acceptor molecule in limiting concentrations, high concentrations of H_2O_2, *and* molecular O_2, a component which is generally neglected but which is almost always present. Reaction of H_2O_2 with ferric chloroperoxidase which generates compound I is considered to initiate the reaction (Figure 3). Compound I can be reduced to compound II by the halogen acceptor molecule, possibly by a radical derived from the halogen acceptor, or even by $O_2^{\cdot-}$. Neidleman and Geigert noted that the chlorination efficiency of "substrates" of chloroperoxidase correlates reasonably well with the degree of "activation" of the hydrogen atom replaced by Cl.[2] In the presence of O_2, under the acidic conditions required for enzyme-mediated chlorination, compounds with an activated hydrogen atom will undergo some degree of autoxidation, catalyzed by low levels of iron typically present in aqueous buffered solutions. As a consequence, low concentrations of the one-electron oxidized substrate radical and $O_2^{\cdot-}$, both reducing species, can be readily formed by nonenzymatic routes. The composition of the reaction mixture will become very complicated once the reaction has been initiated; the redox potentials of the various components will determine the probabilities of reaction of reducing species with various oxidizing species present at different stages of catalysis. For example, compound III could be formed by reaction of the ferric enzyme

and O_2^- under certain conditions. The next step in OCl^- formation is proposed to be conversion of chloroperoxidase compound II to compound III by excess H_2O_2 present in these reactions. Although the point at which Cl^- binds to the enzyme on the path leading to HOCl formation is not clear, we consider that only compound III molecules bound to Cl^- can be activated for productive OCl^- formation by transfer of an electron to the complex. The transferred electron would initiate cleavage of the bound O_2, and immediate transfer of a two-electron deficient oxygen atom to Cl^-; the OCl^- produced would dissociate from the enzyme and undergo rapid protonation to HOCl in the acidic medium. The three-electron reduction of compound III would regenerate ferric chloroperoxidase for another catalytic cycle. This proposed mechanism for chloroperoxidase-catalyzed generation of HOCl involves at least three higher oxidation states of the enzyme and defines essential roles for one-electron reductants in both the conversion of compound I to compound II and the activation of a Cl^--bound compound III species of chloroperoxidase with an effective oxidation state of +6.

The critical reactions in this proposed catalytic sequence, formation of chloroperoxidase compound III and transfer of a single electron to Cl^--bound compound III to initiate oxygen activation, are quite analogous to well-documented reactions in the catalytic cycle of cytochrome P-450.[28] For cytochrome P-450, there is a consensus that the oxygen activation step which immediately precedes substrate oxygenation is transfer of an electron, in a rather specific manner, to a compound III-like ferrous-O_2-substrate complex of the hemeprotein.[28] This reaction would yield the equivalent of a ferric-H_2O_2-substrate complex, which could, in theory, undergo an internal redox reaction to produce a compound I-like species. Such a species has been generally assumed to be the active oxygen transfer agent of cytochrome P-450.[47-49] Although many laboratories have devoted much effort to this question, to the best of our knowldege, no one has been able to demonstrate unequivocally cytochrome P-450 compound I formation with any peroxide.[46,50] The fact that HRP can bind Cl^-,[137] but cannot catalyze oxidation of this particular halide, suggests that compound I of HRP does not contain sufficient energy to oxidize Cl^- (cf. Section IV.A.4.a). Whether the known compound I of chloroperoxidase or the postulated compound I species of cytochrome P-450 would retain a greater percentage of the energy of H_2O_2 reduction for O-Cl or O-C bond formation is not apparent. It could be argued that a concerted cleavage of the HO-OH bond *and* formation of an O-Cl or O-C bond carefully orchestrated by an enzyme would increase the likelihood of efficient transfer of energy from H_2O_2 into the newly formed product bond. However, Ortiz de Montellano has published rather definitive evidence for a nonconcerted mechanism of cytochrome P-450 catalyzed hydroxylation, involving formation of a carbon-centered radical of the hydrocarbon substrate, that collapses rapidly (10^9 s^{-1}) at the active site.[138] It seems unlikely that Cl^- would be similarly oxidized to $Cl^\cdot$ at the more accessible (to solvent) active site of chloroperoxidase. The principal advantage of the proposed three-electron reduction of compound III would be an energetic one: the energy yield of this step would be greater than that available from two-electron reduction of H_2O_2, compound III, or a postulated, transient compound I species. Consequently, the requirement for highly efficient energy transfer from the activated oxygen species to the new O-Y bond could be relaxed somewhat, permitting a nonconcerted reaction, at least in the case of cytochrome P-450.[138] Moreover, a three-electron reduction of compound III would be expected to provide a sufficient excess of energy beyond that required for O-C or O-Cl bond formation to make this reaction in the catalytic cycle irreversible ($\Delta G < 0$).

Although the detailed reaction path of Cl^- oxygenation must be very complex, it is tempting to speculate on structures of transition states on the reaction path. As a consequence of the requirement to conserve the energy of reduction of the O-O bond, significant O-Cl bond formation may develop *prior* to cleavage of the O-O bond. If this occurs, one possible transition state after the transfer of an electron and a proton to the HCl-bound compound III would be an Fe-HOOHCl structure with formal oxidation state of +5. Subsequent cleavage of the O-O bond would produce OCl^- and Fe(III)H_2O. According to this minimal mechanism, compound I and Fe(III)-OCl^- would not be requisite intermediates directly on the path to OCl^-. In contrast to the

mechanism of halide oxidation involving compound I species,[123] the oxygen atom coordinated to the heme iron of compound III becomes reduced to H_2O and remains coordinated to the cation at the end of the cycle. This would appear to be the most favorable path to products, involving minimal rearrangement of atoms in the postulated Fe-HOO·HCl complex. While a similar complex, Fe-HOO·HC-R, can be proposed for the cytochrome P-450 reaction, the detailed reaction paths for transfer of protons, electrons, and an oxygen atom are probably quite different for the two enzymes, due to differences in chemical reactivity and size of the respective oxygen acceptor substrates.

Direct experimental evidence for the detailed mechanism of chloroperoxidase-catalyzed chlorination reactions has been exceedingly difficult to obtain due to the high catalytic activity of the enzyme and the complexity of these reactions. Although most simple dehydrogenation reactions catalyzed by HRP proceed according to the catalytic cycle shown by Equation 13, a few examples of more complicated reaction sequences have been documented. The peroxidase-catalyzed oxidation of both indole-3-acetic acid[86,119] and NADH[84] is known to involve the participation of both O_2 and compound III of the enzyme. Indeed, the peroxidase-catalyzed oxidation of indole-3-acetic acid has been proposed to proceed by two distinct mechanisms, depending upon the enzyme/substrate ratio.[86] This possibility exists because the one-electron oxidized radical of indole-3-acetic acid readily adds O_2, forming a peroxy radical, which can alter the course of the reaction.[119] It is perhaps not entirely fortuitous that reactions considered atypical for peroxidase occur with biologically important compounds, namely, NADH and indole-3-acetic acid, a plant growth regulator which may be the true substrate of this enzyme *in vivo*. The difficulty of elucidating the mechanism(s) of chloroperoxidase chlorination reactions is, we believe, directly related to their complexity. If the proposal that a transient species of chloroperoxidase coordinated to an O_2 species is correct, then more oxidation states of the enzyme participate in chlorination than in typical dehydrogenation reactions of peroxidases. This possibility, together with the chemical reactivity of the reaction product HOCl, would provide ample opportunity for many other enzymatic and nonenzymatic reactions to occur in these systems.

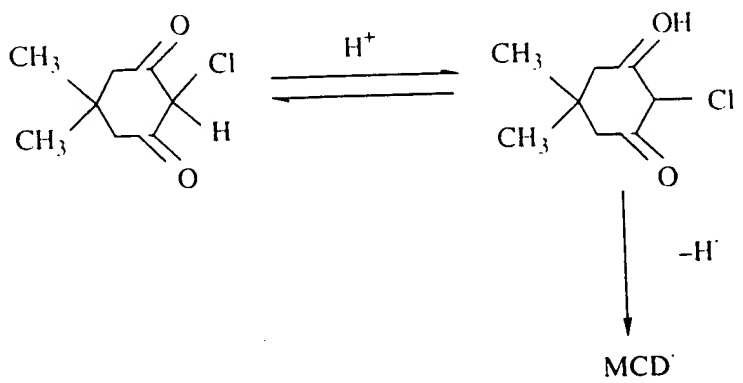
d. Monochlorodimedone (MCD) Chlorination by Chloroperoxidase: Evidence for a Complex Radical Mechanism

One reaction which provides an excellent illustration of the complexity of chloroperoxidase-catalyzed halogenation reactions is halogenation of MCD, used in the standard assay of haloperoxidase catalytic activity.[10] It has been demonstrated that MCD undergoes a facile autoxidation, resulting in a stimulation of O_2 consumption, under the acidic conditions required for enzymatic halogenation.[98] Thus, the reactivity of the MCD free radical with O_2 provided a convenient means of determining if this species could be involved in enzyme-dependent halogenation of the compound. A considerable amount of data, only part of which is summarized here, strongly suggested that the radical is indeed an intermediate in the halogenation reaction. It was shown that chloroperoxidase catalatic activity in the absence or presence of Br^- was inhibited by increasing concentrations of MCD. Thus, MCD was easily oxidized in both systems, probably by a higher oxidation state of the enzyme, in the absence of halide, or by the product of Br^- oxidation. In the absence of halide, the stoichiometry of MCD inhibition of chloroperoxidase-dependent formation of O_2 from H_2O_2 indicated that one-electron oxidation of MCD was highly competitive with oxidation of H_2O_2.[98] Moreover, in the absence of halide, when H_2O_2 was limiting relative to MCD, chloroperoxidase actually catalyzed O_2 *consumption*. This result indicated that virtually all of the H_2O_2 was utilized for MCD oxidation, effectively suppressing the catalatic activity of the enzyme. Under these conditions, increasing the concentration of Br^- or Cl^- (ultrapure grade with minimal Br^- contamination) inhibited chloroperoxidase-dependent O_2 consumption; Br^- inhibited O_2 consumption more effectively at lower concentrations than Cl^-,[98] consistent with other data on the relative rates of enzymatic oxidation of these halides.[10,53]

These results were interpreted in the following way. There is a competition among several reductants, including H_2O_2, MCD, Br^-, and the MCD free radical for reactive oxidants in the system, i.e., higher oxidation states of chloroperoxidase and one or more oxidized bromine species. When H_2O_2 is limiting, MCD and/or Br^- will be the more probable reductants of oxidized enzyme species, and the competitive oxidation of both compounds can likely occur under a range of experimental conditions. Although the rate constants for reaction of the MCD free radical with O_2 and Br_2 are not known, the data indicated that, even at low steady-state Br_2 concentrations, the radical reacted rapidly with Br_2. Thus, low concentrations of Br^- are oxidized sufficiently fast by chloroperoxidase to insure that bromination of the MCD radical competes very effectively with oxidation of the radical by O_2, even in the presence of ambient O_2 concentrations. One ambiguity in the interpretation of these data concerns the rate of generation of the MCD radical, which may be altered in the presence of Br^-/Br_2. Since the MCD radical can be formed by both enzymatic and nonenzymatic routes, one of which was proposed to depend on Br_2, the relative contributions of the various formation and decay reactions of the radical to net O_2 consumption by this species are unknown.

By contrast, the reactions of MCD in a HRP-H_2O_2-Br^- system were shown, in the same study,[98] to depend upon the presence of O_2. In separate reactions conducted in the presence of O_2, HRP catalyzed (1) the H_2O_2-dependent oxidation of Br^- to $Br3^-$,[98] and (2) autoxidation of MCD, with limiting H_2O_2 or without H_2O_2.[98] As mentioned previously, much higher concentrations of HRP and Br^- were required for Br^- oxidation than in a chloroperoxidase system.[98] However, in O_2-containing solutions of HRP and H_2O_2 containing both Br^- and MCD, only minor amounts of brominated MCD were formed; under argon, the same reaction mixture produced near quantitative amounts of brominated MCD.[98] These results indicated that HRP oxidized MCD more readily than Br^- and that, in this system, the MCD radical reacted preferentially with O_2, present at much higher concentrations than Br_2. Only when O_2 was excluded could the concentration of the MCD radical increase sufficiently to trap the low amounts of Br_2 produced by HRP, thus, forming the brominated product. These data provided an explanation for the previously reported failure of HRP to catalyze MCD bromination in air-saturated solutions. The interpretation most consistent with all of the data considered the relative probabilities of: (1) enzymatic oxidation of both MCD and Br^- by each hemeprotein and (2) nonenzymatic radical reactions. Thus, the mechanism proposed for enzymatic halogenation of MCD was a mixed enzymatic/nonenzymatic radical chain reaction involving the MCD free radical as a required intermediate.

This study[98] of enzyme-dependent halogenation of MCD did not establish the identity of the hemeprotein species which participated in catalysis. However, the experimental data strongly suggested that the essential role of the hemeprotein in each system was the generation of free HOBr, which was subsequently converted to Br_2, presumed to be the actual halogenating species.[98] It was proposed that the enzyme-dependent halogenation of MCD occurs by the same radical chain mechanism established for chemical halogenation reactions of MCD and other compounds with X_2:[139]



(17)

$$MCD^{\cdot} + Br_2 \rightarrow MCDBr + Br^{\cdot} \quad (18)$$

$$Br^{\cdot} + MCD \rightarrow MCD^{\cdot} + Br^{-} + H^{+} \quad (19)$$

The energetics of the overall reaction are very favorable for Br_2 or Cl_2 and alkyl radicals derived from compounds, such as MCD, with an activated hydrogen atom.[139] It is important to note that X_2 is considerably more reactive than HOX.[118] Since low concentrations of X_2 exist in the presence of HOX, X^-, and H^+, the rate of substrate halogenation by HOX will be faster in the presence than in the absence of X^-. The ability of X^- to accelerate purely chemical reactions of HOX with MCD[112] and H_2O_2[53] has been demonstrated, in support of this proposal (Section III.D).

If this hypothesis for the mechanism of MCD halogenation by chloroperoxidase and HRP is correct, the number of species present at various times during these reactions can be large. They include, in addition to the reactants added initially, the MCD free radical, produced from the parent molecule by various routes including autoxidation, reaction with X^-, or reaction with a higher oxidation state of the hemeprotein; HOX, X_2, and X^-; O_2 and O_2^- produced by reaction of the MCD radical with O_2; various oxidation states of the hemeprotein catalyst; and the brominated product, as well as a poorly-characterized oxidation product of MCD.[10] It is also clear that there are many possibilities for competing reactions. Consequently, the course of the reaction and the final product(s) may be different under different experimental conditions, as illustrated by different routes of reaction of the MCD radical in the HRP bromination system under argon or air.[98] One example of competing reactions in these systems is given: the MCD$^{\cdot}$ radical can react with at least three components: O_2, another substrate radical, or X_2, in reactions that result in, respectively, either net two-electron oxidation of MCD concomitant with O_2^- formation, or regeneration of fully reduced MCD, or cleavage of X_2 with formation of halogenated MCD and X^-. Many other examples of competing reactions in these systems can be cited. The possibility that low levels of contaminating transition metal ions might participate in some of the solution reactions cannot be excluded, since it has been reported that metal ions influence the mechanism of peroxidase-catalyzed oxidation of indole-3-acetic acid.[86] It is clear that very complicated reaction dynamics will determine which of several possible reaction pathways will be favored at various stages of the reaction, i.e., initiation, maintenance (or steady state), and termination. For example, when Br^- is limiting, the enzyme will utilize MCD as reductant in preference to Br^-. Consequently, the higher concentrations of the MCD free radical produced will react preferentially with O_2 or with another MCD radical instead of Br_2, which may be formed only in very low amounts. Under such conditions, the probability of net oxidation of MCD is increased at the expense of the brominated product. On the other hand, when MCD is limiting relative to H_2O_2 and Br^-, the likelihood of a "futile" cycle is increased, in which the oxidized bromine product reacts with one reactant, H_2O_2, producing O_2 and regenerating the second reactant Br^- (Section III.D).

Since the chemistry occurring in chloroperoxidase-dependent halogenation reactions is very complicated, predicting which enzyme species are present under steady-state conditions would be very difficult, even if all rate constants were accurately known. A few attempts to record the absorbance spectra of chloroperoxidase under steady-state conditions of halogenation have been reported.[10,112] Such experiments are more difficult with chloroperoxidase than with other hemeproteins because of the high catalytic activity of the enzyme. Concentrations of hemeproteins typically employed for measurement of absorbance spectra under steady-state or single-turnover conditions are near 1 μmol/l, considerably larger than the chloroperoxidase concentrations (in the range of nanomoles per liter) required for substantial rates of substrate halogenation. In order to observe a significant fraction of the enzyme in a state other than the ferric form during catalysis, it has generally been necessary to resort to experimental conditions that are quite different from steady-state conditions for halogenation. For example, Thomas reported a compound II-like spectrum of chloroperoxidase, with Soret absorbance near 435 nm, in the

presence of 10 mmol/l each of MCD, H_2O_2, and NaCl.[10] This is a relatively high concentration of H_2O_2, considering the reactivity of chloroperoxidase with H_2O_2, but a low concentration of Cl^- relative to the apparent dissociation constant of 0.2 mol/l at pH 3.0,[15] so these conditions were not optimal for halogenation. Thomas also noted that the rapid, spontaneous decay of chloroperoxidase compound I did not produce a compound II species, as occurs with HRP, or any detectable intermediate other than ferric enzyme.[10] A similar finding was reported by Dunford et al. in a system containing 0.1 mmol/l MCD, 2.4 mmol/l peracetic acid as the peroxidic agent (to prevent catalatic decomposition of the oxidant), and variable Cl^-;[112] at higher Cl^- concentrations, a larger fraction of chloroperoxidase was observed to be in the ferric state. Although this effect of higher Cl^- was interpreted to indicate faster Cl^--promoted decomposition of compound I to yield HOCl,[112] another interpretation is greater inhibition of chloroperoxidase compound I formation by higher Cl^-,[10] as described in Section IV.A.3. Also, in view of the documented complexity of chloroperoxidase-dependent halogenation of MCD with H_2O_2,[98] it is not clear whether MCD halogenation with peracetic acid proceeds by identically the same mechanism as the H_2O_2-supported reaction. In previous studies of MCD halogenation by haloperoxidases and any peroxidic agent, the possible involvement of the MCD radical has been overlooked.[69,112] However, from the published data on the properties of this radical, we conclude that it would be easily generated in all of these systems.

The specific reaction sequence below is proposed to incorporate the hypothesis concerning the role of compound III in Cl^- oxygenation with the experimental evidence for involvement of the MCD radical in the halogenation reaction:

$$\text{E - Fe(III)} + H_2O_2 \longrightarrow \text{compound I} + H_2O \tag{20a}$$

$$\text{compound I} + AH \longrightarrow \text{compound II} + A^{\cdot} \tag{20b}$$

$$\text{compound II} + H_2O_2 \longrightarrow \text{compound III} + H_2O \tag{20c}$$

$$\text{compound III} + AH + HCl \longrightarrow \text{E} - \text{Fe(III)} + H_2O + HOCl + A^{\cdot} \tag{20d}$$

$$HOCl + HCl \longrightarrow Cl_2 + H_2O \tag{20e}$$

$$2A^{\cdot} + Cl_2 \longrightarrow 2\ ACl \tag{20f}$$

$$\text{Net: } 2\ H_2O_2 + 2\ AH + 2\ HCl \longrightarrow 4\ H_2O + 2\ ACl \tag{20g}$$

Equation 20g has the correct stoichiometry for enzymatic halogenation reactions (cf. Equation 1), but was derived from a reaction sequence representing a different mechanism from that previously proposed for these reactions. The net reaction Equation 20g can actually be considered to result from a different sequence of reactions, shown below. One reaction of this sequence, Equation 21b is remarkably similar to the general equation for cytochrome P-450 catalyzed oxygenation reactions (cf. Equation 5):

$$2\ H_2O_2 \longrightarrow O_2 + 2\ H_2O \tag{21a}$$

$$O_2 + 2\ AH + HCl \longrightarrow H_2O + HOCl + 2\ A^{\cdot} \tag{21b}$$

$$HOCl + HCl \longrightarrow Cl_2 + H_2O \tag{21c}$$

$$2\ A^{\cdot} + Cl_2 \longrightarrow 2\ ACl \tag{21d}$$

Equations 21a to 21b suggest that Cl^- oxygenation involving compound III of chloroperoxidase can be considered as an activation of O_2, with the O_2 provided by a catalatic-like decomposition of H_2O_2. The O_2 produced from H_2O_2 is actually a heme-coordinated O_2 species in a compound III, or dioxygen-ferrous species, of the enzyme. According to the reaction sequence of Equation 21, enzymatic Cl^- oxygenation can be formally considered as a monooxygenation reaction. Electrons required for reduction of the second oxygen atom to H_2O are supplied, one each, by two molecules of the terminal halogen acceptor AH. According to this hypothesis, AH serves two functions in these reactions: as an electron donor, similar to the role of NAD(P)H in cytochrome P-450-catalyzed oxygenation reactions, and as the terminal halogen acceptor. The electron donor function of AH in oxygen activation is quite consistent with the observation of Neidleman and Geigert[2] that halogen acceptor "substrates" of haloperoxidases, in general, have an activated hydrogen atom. Since this property will facilitate autoxidation, it seems likely that efficiency of enzymatic halogenation of a series of compounds might correlate with their susceptibility to autoxidation under optimal conditions for halogenation. However, data demonstrating such a correlation have not been published. The analogy between enzymatic activation of O_2 for oxygenation reactions and nonenzymatic autoxidation reactions, which can be considered as a variant of oxygen activation mediated by trace levels of iron or copper, is useful, since the mechanisms of the two reactions may be more similar than has been appreciated. This analogy reinforces the idea that the requisite oxidized enzyme species on the direct path of oxygen activation contains a bound dioxygen species that is transformed into an energetic peroxidic species in the transition state. Such a mechanism is more compatible with the structure of compound III than the structure of compound I.

The proposed involvement of a radical species $A\cdot$ of the terminal halogen acceptor, Equation 20, is also consistent with current thinking about the role of radical species of oxygen acceptors in cytochrome P-450-catalyzed oxygenation reactions.[50] Although nonradical pathways for ACl formation are not excluded, direct reaction of the radical $A\cdot$ with Cl_2, via the reaction sequence Equation 17 to 18, would be very fast.[98,112] The presence of even low levels of a very reactive radical would provide an effective trap for Cl_2, which would protect the enzyme from destruction by oxidized halogen species. There is now considerable evidence that the "activated" oxygen produced during cytochrome P-450 monooxygenation reactions can abstract an electron or hydrogen atom from the oxygen acceptor or electron donor substrate bound at the active site, and that the organic radicals so produced, which may have only a transient existence at the active site, are directly on the path to products.[50] However, experimental evidence for a compound I species of cytochrome P-450 is nonexistent, although much effort has been expended to identify such a species.[49,50] The evidence and arguments that such a species would have sufficient energy to abstract an *unactivated* hydrogen atom, as occurs with certain aliphatic hydrocarbons, are inconclusive. A partially reduced O_2 species associated with the heme of cytochrome P-450 would have a more positive redox potential than the hypothetical compound I species and, thus, would be a more attractive candidate for abstracting an unactivated hydrogen atom from an oxygen acceptor substrate.

Elucidating the molecular details of cytochrome P-450-mediated activation of O_2 via a compound III, or ferrous dioxygen, species that leads to a radical intermediate of the organic substrate has proved to be a difficult experimental problem. The very hydrophobic nature of the active site of cytochrome P-450, which has been confirmed by X-ray crystallography[33] and by data on the reactivity of the heme group during turnover conditions,[50] must play an important role in this critical step of catalysis. We believe that the many similarities of the heme structures of chloroperoxidase and cytochrome P-450 relate to a similar functional requirement of both enzymes: formation of an enzyme intermediate more highly oxidized than a compound I species, which can oxidize Cl^- or aliphatic hydrocarbons, respectively, at catalytically significant rates. This statement implies that thermodynamic constraints on these very energetic oxidation reactions define rather stringently the immediate heme environments necessary for the efficient

catalytic functioning of both hemeproteins. As a consequence, it can be postulated that the kinetic and thermodynamic properties of such energetic multicenter reactions occurring at the heme active sites of these enzymes may not be strictly independent, but this idea requires additional experimental support.

Finally, we speculate on differences in the mechanism of the critical oxygen activation and product formation steps of the two hemeproteins. The arguments for such differences are based on: (1) marked differences in the nature of the oxygen acceptor substrates of the enzymes; (2) differences in their active site protein structures;[3,33,34] and (3) differences in the properties of compound III species of the proteins.[28,43,132,133,136] Concerning the last point, the absorbance spectrum of ferrous dioxygen cytochrome P-450[136] resembles closely those of the oxygen transport hemeproteins which bind O_2 reversibly.[140] However, the absorbance spectrum of chloroperoxidase compound III is more similar to those of other peroxidase compound III species.[132,133] The evidence from resonance Raman data for more electron density, and a greater degree of "activation" of O_2, in HRP compound III than in oxy-myoglobin[131] suggests that the predominant, or average, resonance forms of compound III species of chloroperoxidase and cytochrome P-450 may differ: the former best represented by $Fe^{3+}\cdot O_2^-$ and the latter by $Fe^{2+}\cdot O_2$. Figure 4 depicts hypothetical mechanisms for the oxygen activation reactions of chloroperoxidase and cytochrome P-450, diverging from compound III species, which have, on average, different resonance structures. This difference in resonance structures of the two compound III species is likely a necessary consequence of differing reactivities of the oxygen acceptor substrates of the two enzymes. The rate-limiting step of each catalytic cycle is considered to be the same: transfer of an electron to a complex of compound III with the oxygen acceptor. This is definitely established for cytochrome P-450,[28] and appears to be a reasonable assumption for chloroperoxidase, based on relative rates of chlorination of various compounds[2] and on the mechanism which we have proposed. For chloroperoxidase, electron transfer to a Cl^--bound compound III species with $Fe^{3+}\cdot O_2^-$ resonance form is suggested to generate a heme-associated peroxide with an unusual or deficient protonation state, which undergoes heterolytic cleavage assisted by the proximal Cl^-. The transient activated heme-peroxide complex would have effective oxidation state of +5 and would be directly converted to the native ferric enzyme upon release of OCl^-. Controlling the rate of OCl^- formation by electron transfer from the most probable terminal halogen acceptor would be highly advantageous, since it would protect both the enzyme and essential cellular components from inactivation and damage by HOCl that would occur in the absence of a legitimate target for chlorination. Neither compound I nor compound II is considered to be a required intermediate in the decay of the Cl^--bound activated complex; but one or both species might arise in aborted reactions, if Cl^- should dissociate before oxygen transfer were accomplished. This should be unlikely under optimal experimental conditions for chlorination. However, under less favorable conditions, for example, as one of the reactants becomes limiting, the rate of OCl^- formation will decrease as other reactions become relatively more important in the termination phase.

The proposal for the decay of substrate-bound ferrous-dioxygen cytochrome P-450 initiated by transfer of an electron to the $Fe^{2+}\cdot O_2$ complex, shown in Figure 4, is somewhat more complicated. Electron transfer produces an O_2^- species, coordinated to the heme and possibly protonated, that abstracts a hydrogen atom from the organic substrate. Two subsequent stages in the postulated transition state are depicted in Figure 4: homolytic cleavage of the O-O bond and recombination of the $^{\cdot}OH$ and carbon-centered radicals at the active site. Since the precise sequence of events in the decay of the activated complex is not readily amenable to direct experimental test, it may differ in certain details from those shown. However, the mechanism proposed must be consistent with published data on these reactions and on different properties of the compound III species of chloroperoxidase and cytochrome P-450. One essential difference between chloride oxygenation by chloroperoxidase and hydrocarbon oxygenation by cytochrome P-450 appears to be the involvement of a short-lived radical intermediate of the

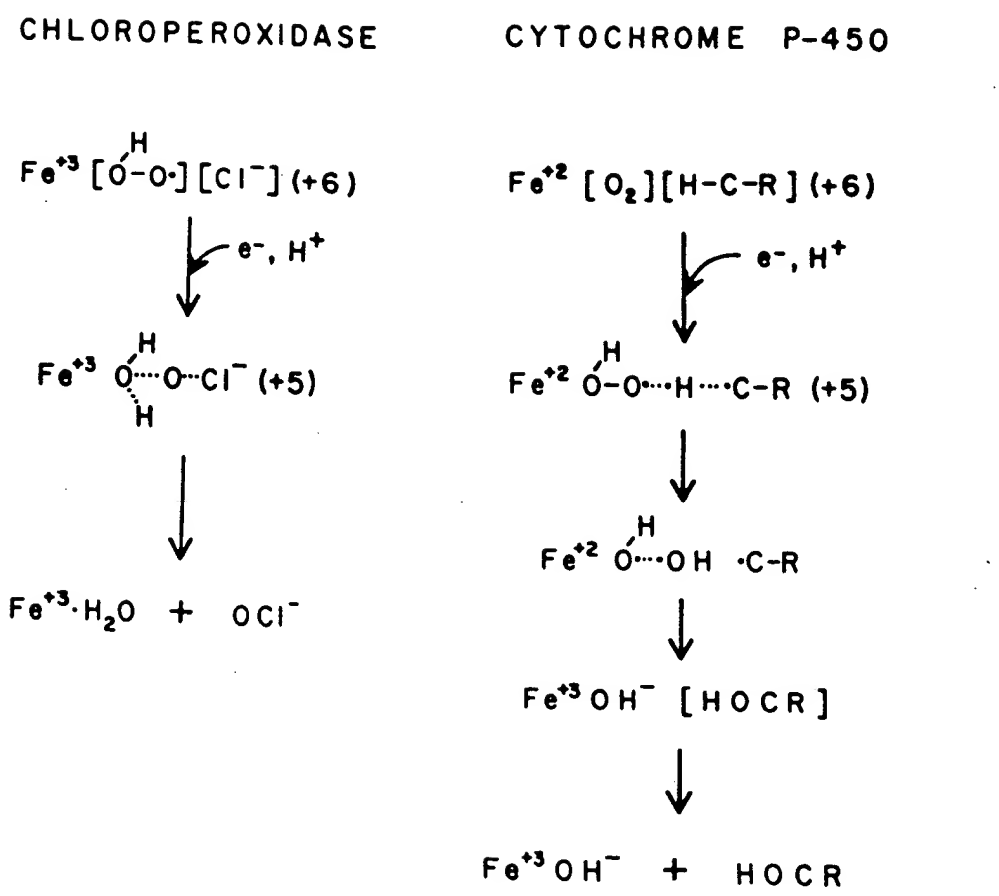


FIGURE 4. Compound III species of chloroperoxidase and cytochrome P-450: postulated mechanisms of reductive decay to oxygenated products. Each compound III species is complexed to both of its substrates; brackets designate highly specific interactions of the bracketed compounds with the respective enzyme active site. The major resonance form of chloroperoxidase compound III is proposed to be a ferric-superoxide (protonated) complex, by analogy with other peroxidase-like compound III species. Transfer of an electron to this species converts it to an unusual transient ferric-peroxidic complex with oxidation state of +5. The O–O bond breaking and O–Cl^- bond formation processes are considered to be heterolytic. By contrast, the +6 oxidation state of cytochrome P-450 is postulated to be predominantly a ferrous-dioxygen species, and the various bond breaking (H–C– and O–O) and bond formation (HO–C^-) processes are depicted as having considerable homolytic character. For both decay routes, the timing of proton transfer is unknown, but is considered to be critical for productive completion of the cycle.

hydrocarbon substrate, which remains at the active site until it has been converted to the final oxygenated product.

It is appropriate to ask whether the mechanisms proposed to account for the catalytic functions of chloroperoxidase and cytochrome P-450 shed any light on the evolutionary origins of hemeprotein functional diversity. The hypothesis for chloroperoxidase-catalyzed chlorination accounts for the experimental observations that the best terminal halogen acceptor molecules (AH) of this enzyme have an activated hydrogen atom.[2] This property not only promotes compound III formation in the presence of excess H_2O_2 and the decay of the "activated" Cl^--compound III complex, but likely increases the probability of trapping the oxidized chlorine species by reaction with the radical species of the halogen acceptor. It was suggested earlier that the degree of hydrogen atom activation of the terminal halogen acceptor may be an important control over the rate of formation of OCl^-. However, since many essential cellular components can be halogenated by the oxidized halogen products of haloperoxidases, another important control over the potentially lethal activity of these enzymes appears to be their localization, in secretory granules, or in the extracellular space.[4,8,12,26] When experimental

conditions for oxidation of halide exist and a target is present, these enzymes release OX^- into the local environment, the composition of which determines specificity of halogenation. By contrast, the monooxygenase activity of cytochrome P-450 is a highly regulated function which may have evolved from a more primitive chloroperoxidase-like activity. Properties of cytochrome P-450 which differ substantially from chloroperoxidase include: (1) a hydrophobic protein region near the heme[33,34] for specific binding of the oxygen acceptor substrate, which insures that the substrate traps the "active" oxygen efficiently and stereospecifically and, thus, precludes release of reactive radical intermediates from the enzyme;[1,38] and (2) an associated system of electron transport proteins, which supplies two electrons from NAD(P)H for complete reduction of O_2,[28] and, more importantly, prevents a nonproductive, possibly destructive reaction of the "active" oxygen with the pyridine nucleotide, which could occur if the latter were bound directly to cytochrome P-450. The monooxygenase function of cytochrome P-450 appears to have evolved for *in situ* generation of a two-electron reduced, highly energetic O_2 species distinct from H_2O_2 in its reactivity at the active site of this hemeprotein. It is possible that many of the diverse functions of hemeproteins related to binding, reduction, and activation of O_2 and H_2O_2 evolved from a common primitive heme-O_2 complex with structure and chemical reactivity similar to those of a compound III species.

The mechanisms of Cl^- oxidation and Br^- oxidation by chloroperoxidase need not necessarily be the same. Compound III may be a requisite intermediate in the catalytic cycle only when an enzyme species more highly oxidized than compound I is needed to generate the energy for O-X^- bond formation. Because of the thermodynamic constraints on Cl^- oxidation, the mechanism of O-Cl^- formation involving compound III may be specific for this halide. Since the half-life of compound I of chloroperoxidase is decreased dramatically by near-stoichiometric concentrations of Br^-,[10] since Br^- can be oxidized by a HRP-H_2O_2 system,[52] and since HRP compound I has a sufficiently positive redox potential to oxidize Br^-, chloroperoxidase-catalyzed Br^- oxidation may occur primarily via a catalytic cycle involving compound I.

Direct evidence for the identity of the active Cl^--oxidizing species of chloroperoxidase, whether compound III, or a complex of the ferric enzyme with H_2O_2 and Cl^-, or perhaps a compound I-like species containing a single, highly activated oxygen atom, is presently lacking. Since compound III of chloroperoxidase has been characterized only in the absence of Cl^-, critical questions about the effect of Cl^- on the formation and stability of this species must be addressed, in order to determine if this species plays any role in Cl^- oxidation. Can compound III be formed by any method from the Cl^--bound enzyme? Does Cl^- accelerate compound III decay at a rate consistent with the overall rate of chlorination or must an electron be provided to activate a compound III-Cl^- complex? It could be argued that the absorbance spectrum of a Cl^--bound form of compound III of chloroperoxidase should resemble more closely that of the productive ferrous-O_2-substrate complex of cytochrome P-450; the latter species slowly decomposes to O_2^- and ferric enzyme in the absence of the necessary electron and effector molecule to yield product.[1,36] However, oxycytochrome P-450 is quite unstable in the absence of substrate.[29] If Cl^- binding to chloroperoxidase compound III cannot account for the different spectral properties of this species and the ferrous-O_2-substrate complex of cytochrome P-450, an alternative explanation is a difference in the protonation states of these complexes. The unusually low pH optima of chloroperoxidase-dependent chlorination reactions, near pH 3 or 4, are thought to reflect the participation of an acidic group with $pK_a < 2$ in Cl^- binding to the ferric enzyme.[15] However, protonation and/or deprotonation of this group might be involved in catalysis in other ways, for example, by controlling the binding of Cl^- to compound III or by providing a proton to balance the charge of an electron transferred during the reaction. It is apparent that elucidation of specific details of the reaction mechanism of HOCl formation by chloroperoxidase will require, at a minimum, clarification of a possible catalytic role of compound III, or other intermediate containing bound dioxygen, more information about the interaction of Cl^- with the enzyme species that activates oxygen for substrate oxygenation, and

delineation of the sequence of transfer of electrons and protons necessary to complete the catalytic cycle.

e. Evidence for Involvement of compound III in Myeloperoxidase Function

Although a comprehensive discussion of halogenation reactions catalyzed by other enzymes cannot be included in this review, it is informative to examine some of the recent pertinent data published for selected reactions of this class. Myeloperoxidase has been extensively characterized; this hemeprotein haloperoxidase is responsible for HOCl formation and the resultant microbicidal activity of stimulated neutrophils.[4-6] As one of only three hemeproteins shown to catalyze chlorination reactions with H_2O_2 and Cl^-, myeloperoxidase differs from chloroperoxidase in several respects; therefore, a comparison of the physical properties and catalytic functions of the two enzymes is of considerable interest. Myeloperoxidase is a tetrameric protein of molecular weight approximately 146,000 Da, containing 3% carbohydrate;[141] the subunit structure has been controversial, since the number and mass of the subunits depend upon the degree of control of proteolysis during purification of the protein.[142,143] The protein contains two unique, apparently identical heme groups, covalently linked to the two larger identical subunits, with mass of 55,000 Da.[142] Although the porphyrin structure of the myeloperoxidase heme groups has not been conclusively identified, its spectral properties resemble a chlorin structure more closely than protoporphryin IX.[144,145] The pH optima of myeloperoxidase chlorination reactions are typically 1 to 3 pH units higher than for the same reactions catalyzed by chloroperoxidase, which have pH optima near pH 3 or 4.[2] The MCD chlorination activity of the neutrophil enzyme is about 1% of that of chloroperoxidase.[65,117] The lower chlorination activity of myeloperoxidase would appear to be an evolutionary compromise for a hemeprotein chlorination catalyst that must function in a more physiologic pH range. The novel experiments of Harrison and Schultz with immobilized myeloperoxidase, cited earlier, provided the first direct evidence that free HOCl and Cl_2 are products of myeloperoxidase-catalyzed oxidation of Cl^- by H_2O_2.[5] Many studies have documented that both chemical chlorination reactions with HOCl and myeloperoxidase-containing chlorinating systems generate identical products.[2] The experimental evidence for HOCl formation by myeloperoxidase has led to studies in which the relative rates of reaction of HOCl with various endogenous and exogenous biological components, including nonsteroidal anti-inflammatory agents and other drugs, have been measured, as an attempt to determine the most probable targets of myeloperoxidase action in the vicinity of stimulated neutrophils.[129,146,147]

Concerning the catalytic cycle of myeloperoxidase functional in Cl^- oxygenation, certain features have been established, but, as with chloroperoxidase, the reactive enzyme species responsible for oxygen atom transfer to Cl^- has eluded positive identification. The reaction of myeloperoxidase with H_2O_2 produces a species resembling compound I species of other hemeproteins in its spectral properties and reactivity.[148] This species has a half-life of about 100 to 200 ms at pH 7.0 to 7.5,[149] and decays to compound II.[148,149] Thus, the relatively greater stability and mode of spontaneous decay of myeloperoxidase compound I are more similar to other peroxidases than to chloroperoxidase. Complete formation of myeloperoxidase compound I requires a 25- to 50-fold excess of H_2O_2, relative to the hemeprotein,[148,149] whereas most peroxidases react stoichiometrically with H_2O_2 to form compound I. The different behavior of myeloperoxidase has been attributed to an equilibrium reaction of H_2O_2 with the enzyme, although more recent data suggest that the hemeprotein has a significant true catalatic activity when H_2O_2 is not in great excess.[103] Chloride binds to ferric myeloperoxidase as an axial ligand and inhibits reaction of the hemeprotein with H_2O_2.[128] It has been proposed that binding of H_2O_2 and Cl^- to myeloperoxidase are mutually exclusive, reflecting the participation of a common acidic group on the protein: Cl^- binds to the protonated form, and H_2O_2 to the unprotonated form.[128]

A detailed study of myeloperoxidase function published by Winterbourn et al. has raised

interesting questions about the catalytic cycle of this hemeprotein under physiologic conditions.[150] The spectral properties and reactivity of myeloperoxidase associated with, and also added to the medium of, phagocytosing neutrophils were characterized. The data revealed rather complicated behavior of the enzyme which suggested that the prevailing H_2O_2 concentration determines the specific catalytic cycle and ultimate function of the enzyme.[150] An unexpected result was that myeloperoxidase associated with neutrophils stimulated by various means existed almost completely as compound III. The extent of compound III formation was shown to reflect the relative rates of formation of O_2^- and H_2O_2, since exogenous myeloperoxidase added to the phagocytosing cells was converted to compound III to a degree that depended on the relative amounts of O_2 and H_2O_2 present initially.[150] In experiments with the purified hemeprotein, autoxidation of compound III to the ferric enzyme was quite slow, with a $t_{1/2}$ of 12 min at 25°C. The rate of autoxidation was not altered by the presence of Cl^-, H_2O_2, or catalase, but was influenced as expected by either superoxide dismutase (faster rate) or an O_2^- generating system (slower rate).[150] However, these data on the sluggish reactivity of myeloperoxidase compound III under single-turnover conditions contrasted with other data suggesting that this form of the enzyme is quite reactive under rapid-turnover conditions of catalysis. For example, as a catalyst of MCD chlorination, preformed compound III was shown to be indistinguishable from the ferric enzyme over a wide range of H_2O_2 concentrations. At H_2O_2 concentrations greater than about 0.5 mmol/l, both forms of the enzyme exhibited identical behavior, namely, a decrease in the extent of MCD chlorination and an increase in H_2O_2-decomposition activity.[150] Significantly, MCD chlorination activity of myeloperoxidase was demonstrated in the presence of an O_2^- generating system (xanthine/xanthine oxidase), which converted about 70% of the enzyme to compound III. Moreover, addition of superoxide dismutase to this system to inhibit compound III formation did not increase the rate of MCD chlorination.[150] This result provided additional evidence for similar reactivity of the ferric and compound III forms of myeloperoxidase as catalysts of MCD chlorination.

Based on these data, and numerous control experiments not described here, Winterbourn et al. proposed[150] that myeloperoxidase in stimulated neutrophils exists as a compound III species, which can function in either chlorination or H_2O_2 decomposition, depending on the H_2O_2 concentration. The results indicated that at low H_2O_2 levels (<200 μmol/l), H_2O_2 was utilized for HOCl formation and chlorination of MCD occurred with nearly 100% efficiency; at H_2O_2 concentrations in excess of 0.5 mmol/l, H_2O_2 was decomposed to O_2. In effect, the role proposed for myeloperoxidase compound III was as a reservoir of O_2^-, which could reduce compound II, produced spontaneously from compound I in the presence of excess H_2O_2. This proposed electron transfer between compounds III and II of the hemeprotein would, thus, regenerate two molecules of the ferric enzyme and a molecule of O_2, in an apparent futile cycle for the phagosome. Ferric myeloperoxidase could then cycle repeatedly through these reactions, resulting in net decomposition of H_2O_2 or, under conditions of limiting H_2O_2, could enter the Cl^- oxidation catalytic cycle via compound I formation.[150] It appears from this study that myeloperoxidase compound III is not an inactive species, but may play some role in the functioning of the enzyme under physiologic conditions. However, not all published data on myeloperoxidase compound III are consistent with this interpretation. For example, Cuperus et al.[151] reported the inhibition of myeloperoxidase chlorination activity (toward MCD) by D-penicillamine and suggested that this sulfhydryl compound promotes accumulation of compound III as an inactive form. Their arguments were based on effects of D-penicillamine on myeloperoxidase spectral properties under single-turnover conditions which were quite different from those employed for studies of the catalytic properties of the enzyme.[151] The mechanism proposed for these observations required that D-penicillamine serve as an electron donor to several distinct oxidants in the system, most importantly, to O_2, forming O_2^-, which reacted with ferric myeloperoxidase to form compound III.[151] We conclude, however, that the limited data presented by Cuperus et al. for a *catalytic* system, which contained nanomole per liter concentrations of the enzyme and

also micromole per liter concentrations of ascorbate for some unexplained reason, provided insufficient evidence for myeloperoxidase compound III as the species present in the D-penicillamine-inhibited chlorination system.[151] The catalytic system studied by Cuperus et al.[151] was capable of generating radical species of MCD, D-penicillamine, and ascorbate in the same reaction. Although this complexity was not acknowledged, it certainly would alter the interpretation of the data. We note that Winterbourn et al.[150] did not suggest any direct involvement of compound III of myeloperoxidase in Cl^- oxidation. Moreover, they did not report the effect of Cl^- on myeloperoxidase compound III decay in the presence of MCD, and failed to consider that MCD can be oxidized to a radical species that might participate directly in the reaction.[150]

Although the intriguing data of Winterbourn et al.[150] on myeloperoxidase compound III are consistent with the role proposed in this review for chloroperoxidase compound III in catalysis of Cl^- oxidation, that role can only be defined by experiments with chloroperoxidase. It is possible that the two enzymes oxidize Cl^- by quite different mechanisms, since they display many differences in physical properties, optimal conditions for catalysis, and even the catalytic rates of Cl^- oxidation. Indeed, such differences could be responsible for reported differences in the stability of compound III species of the two hemeproteins.[132-134,148,150] Clearly, more detailed comparative studies of the Cl^- oxidation activities of chloroperoxidase and myeloperoxidase are needed to resolve these questions.

f. Is Intramolecular Electron Transfer Involved in the Catalytic Functions of Chloroperoxidase and Myeloperoxidase?

Both heme-containing subunits of myeloperoxidase appear to behave independently and identically under single turnover conditions.[103,148,149] However, it seems possible that, under certain experimental conditions, the heme groups on the same molecule could be in different oxidation states for a finite time interval, a situation that should increase the probability of intramolecular electron transfer. Transfer of an electron from a ferric heme group to a compound I heme species on the same myeloperoxidase molecule would convert both heme groups to compound II species, which could explain the spontaneous formation of myeloperoxidase compound II in the presence of a moderate excess of H_2O_2.[149] The possible function of the second redox center, i.e., another heme group of myeloperoxidase or the Mn^{2+} ion of chloroperoxidase, in the catalytic actions of these enzymes remains to be defined. There would be obvious advantages of having a mechanism for intramolecular electron transfer in these hemeproteins which can generate heme intermediates with redox potentials sufficiently positive to oxidize Cl^- to HOCl. Appropriately timed electron transfer to reactive oxidizing species, associated with the heme group or released as reaction products during catalysis of Cl^- oxidation, might prolong the useful lifetimes of these catalysts, which are known to be inactivated by their oxidized chlorine products in the absence of a halogen acceptor molecule.[5,7,10,53]

B. OXYGENATION REACTIONS

1. Alkene Epoxidation

The ability of chloroperoxidase to catalyze halide-independent oxygenation of organic compounds, in contrast to the well-characterized Cl^- oxygenation reaction, was demonstrated only recently for selected alkenes[17,18] and a limited series of organosulfur compounds.[19,109,153] Since these reactions are among the broad range of oxygenation reactions catalyzed by cytochrome P-450, these studies provided the first definitive evidence for a functional similarity between chloroperoxidase and cytochrome P-450. For each reaction type, a direct comparison of the reactions catalyzed by chloroperoxidase and either cytochrome P-450 or other peroxidases was also undertaken. Alkene epoxidation reactions catalyzed by chloroperoxidase in the presence of excess H_2O_2 were first described by Geigert and colleagues.[17] It was reported that yields of epoxide were considerably greater with the aromatic alkene styrene than with certain aliphatic alkenes examined and that several other peroxidases, including myeloperoxidase,

could not catalyze this reaction.[17] The yield of styrene oxide, approximately 40% under the experimental conditions reported, did not vary over the pH range 3.0 to 6.0, but at pH 3.0, the product was hydrated moderately fast to the corresponding glycol. The styrene oxidation reaction of chloroperoxidase was actually discovered as a side reaction occurring during the bromination of styrene by this enzyme.[17] These data on styrene reactivity in chloroperoxidase-containing systems are consistent with chemical evidence for hydrogen atom activation of styrene.[155]

Ortiz de Montellano and coworkers[18] undertook a more detailed study of styrene epoxidation catalyzed by chloroperoxidase, in an effort to relate the mechanism of this reaction to the cytochrome P-450-catalyzed O_2-dependent epoxidation of styrene. At pH 6.0 in the presence of a high concentration of H_2O_2, chloroperoxidase catalyzed a rapid burst of oxidation of the alkene to approximately equivalent amounts of styrene oxide and phenylacetaldehyde.[18] The cytochrome P-450-catalyzed reaction produced predominantly styrene oxide and only very low amounts of phenylacetaldehyde.[18] The most significant findings relevant to mechanism were (1) with both enzymes, the stereochemistry of styrene oxide produced from specifically deuterated *trans*-styrene was retained and (2) the oxygen atom of the product of the chloroperoxidase-catalyzed reaction originated solely from H_2O_2.[18] By contrast, HRP-catalyzed oxidation of styrene to styrene oxide by H_2O_2, which had been previously characterized by the same laboratory, requires molecular O_2 and a cosubstrate, such as reduced glutathione, and results in loss of stereochemistry of the product.[156] It was proposed that the primary function of HRP in styrene epoxidation is generation of a thiyl radical from reduced glutathione, and that the thiyl radical mediates activation of O_2, with no direct involvement of the enzyme and, thus, no stereochemical constraints on the product epoxide.[156] Two possible mechanisms of O_2 activation were proposed, involving either a thiyl peroxy radical or an alkyl peroxy radical produced from a thiyl radical adduct of styrene.[156] Clearly, the chloroperoxidase-catalyzed conversion of styrene to styrene oxide resembled more closely the cytochrome P-450-catalyzed reaction than the HRP-mediated "cooxidation" process. Ortiz de Montellano proposed that chloroperoxidase compound I directly transferred an oxygen atom to styrene.[18] Based on these results and other data from the author's laboratory related to the mechanism of peroxidative one-electron transfer reactions of HRP,[157,158] the reactivity difference between compounds I of chloroperoxidase and HRP was attributed to different degrees of access of oxidizable substrates to the Fe(IV) coordinated oxygen atom.[18] Electron donor substrates, it was argued, could gain access only to the heme "edge" of HRP compound I and, after oxidation, diffused into the solution as free radicals.[18] The greater access of substrates to the heme iron of chloroperoxidase allowed both peroxidative and oxygen transfer reactions of compound I, depending upon the preferred reaction route of the substrate.[18]

This proposal of Ortiz de Montellano[18] is quite consistent with the many elegant studies of this group which have delineated the importance of steric factors in oxidation reactions catalyzed by various hemeproteins.[157,158] Some form of chloroperoxidase must directly mediate the stereospecific transfer of an oxygen atom from H_2O_2 to styrene,[18] in contrast to the reaction catalyzed by HRP,[156] but the identity of this oxidized chloroperoxidase intermediate has not been established. However, the chloroperoxidase-catalyzed oxygenation of styrene poses the same dilemma as Cl^- oxygenation: does a compound I intermediate, with a single oxygen atom coordinated to the heme iron, contain sufficient energy to oxidize an alkene to an epoxide, even when the oxygen acceptor (styrene) is an aromatic alkene with activated hydrogen atoms? This question presumes that the steric factors controlling access of a given oxygen acceptor to compound I or compound III of the same hemeprotein are not significantly different. Experimental data which would distinguish the relative contributions of energetic and steric constraints on the reactivity of compound I species are presently limited. The styrene oxygenating agent produced in the HRP-H_2O_2 system, which required the participation of both O_2 and reduced glutathione, was proposed to be one of two possible peroxy radicals.[156] Peroxy radicals with an

intact O-O bond should be more energetic oxygen transfer agents than compound I of HRP, which contains only part of the energy originally in the peroxide oxidant. The role of peroxy radicals in formation of epoxides from polycyclic hydrocarbons has been well documented, but the mechanism of such reactions is not yet established.[46] The oxygen atom transfer reactions of HRP appear to be limited to easily oxidized inorganic compounds, such as I^-, Br^-, and SCN^-, and exclude Cl^-, which is more difficult to oxidize. The upper limit of the energy of compound I of HRP defined by this series of oxygen acceptor substrates is consistent with the measured redox potential of this intermediate.[122] However, the peroxy radical-mediated epoxidation of alkenes and other highly unsaturated compounds suggests that an oxidized species of chloroperoxidase or cytochrome P-450 containing a bound dioxygen species would have sufficient energy not only to form the epoxide bond, but also to confer a negative ΔG value on this critical step, thus making the overall reaction irreversible.

Given the chemical evidence for activation of styrene hydrogen atoms for abstraction,[155] it is proposed that styrene can donate an electron to compound III of chloroperoxidase, as was proposed for other terminal halogen acceptors with activated hydrogen atoms. Halide, if present, would be the preferred oxygen acceptor. The oxidized halogen product and the styrene free radical could then react nonenzymatically, as proposed in Equation 20, to form the halogenated styrene product.[2] In the absence of halide, the activated hemeprotein-O_2^{2-} complex (+5 oxidation state) formed by transfer of an electron to compound III would likely dissociate from the styrene radical and subsequently collide with a molecule of styrene, resulting in formation of the epoxide product and regeneration of the ferric enzyme. However, the unstable activated hemeprotein-O_2^{2-} complex might also undergo a unimolecular decay with low probability prior to its reaction with styrene. Such an internal, or "self", oxidation of the hemeprotein to unidentified product(s) could account for the reported inactivation of the catalyst during the reaction.[18] The proposal of a common (or very similar) hemeprotein +5 intermediate, resulting from one-electron reduction of chloroperoxidase compound III, in both halide oxygenation and styrene epoxidation reactions of this enzyme is consistent with the finding that styrene oxide is a minor product in the enzymatic bromination system.[17]

According to this proposal, styrene serves two functions, i.e., electron donor and oxygen acceptor, in chloroperoxidase-mediated oxidation of this compound. This is consistent with formation of substantial amounts of a second product, phenylacetaldehyde, in this system.[18] The mechanism of formation of phenylacetaldehyde is less clear. It was reported that the rate and extent of formation of phenylacetaldehyde from styrene in this system were similar to those for styrene oxide.[18] It is possible that the styrene free radical can donate an electron to H_2O_2, analogous to a Fenton reaction in which the reductant is an organic radical rather than Fe^{2+}.[92] However, with the styrene radical as electron donor, the HO^- product might never exist free in solution, but would likely be trapped during its formation by the organic molecule. A plausible reaction scheme is depicted below:

$$\mathrm{Ph{-}\overset{\cdot\cdot}{C}H{-}C} + H_2O_2 \rightarrow \mathrm{Ph{-}\overset{\cdot\cdot}{C}H{-}\overset{\cdot\cdot}{C}{-}O{-}OH} \xrightarrow{e^-} \mathrm{Ph{-}CH_2{-}C{=}O} + OH^- \qquad (22)$$

The possibility that adventitious iron participates in this reaction cannot be completely eliminated. Since one-electron reduction of compound III by styrene would produce a heme-O_2^{2-} species, with +5 oxidation state and unknown protonation state, a reaction (analogous to Equation 22) of the newly generated heme-bound peroxide species and the styrene radical might occur with low probability prior to dissociation of the styrene radical. We note that one-electron reduction of compound III would likely be the rate-limiting step for generating both the enzymatic epoxidizing agent and the styrene radical. Whether the styrene radical reacts with free

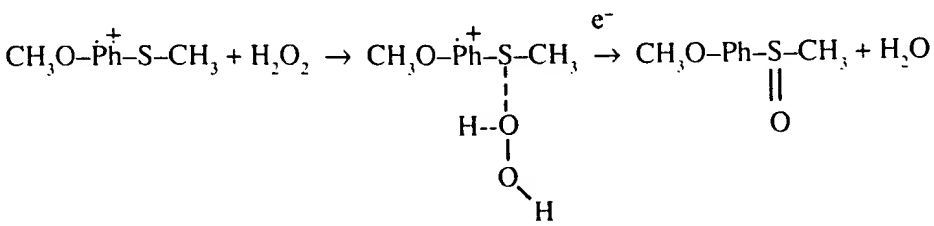
or heme-associated peroxide, this proposal is consistent with the data indicating similar rates and extents of formation of the two products. However, other routes of formation of phenylacetaldehyde are not excluded by the available data. These proposed reaction paths for formation of styrene epoxide and phenylacetaldehyde from styrene in the chloroperoxidase system are working hypotheses that incorporate much of the published experimental data. However, more elaborate, critical experimental tests are required in order to verify the mechanistic details of these proposals.

2. Sulfur Oxygenation

The S-oxygenation of sulfides to form sulfoxides is another class of oxygenation reactions catalyzed by cytochrome P-450.[152] Chloroperoxidase has been shown by two different laboratories to catalyze such reactions, with H_2O_2 as oxidant.[19,153,154] Kobayashi et al. demonstrated that, with chloroperoxidase as catalyst, the oxygen atom of the sulfoxide product of *p*-methylthioanisole arose exclusively from H_2O_2, and that the degree of chirality of the sulfoxide product of this compound was very similar to that produced with cytochrome P-450.[19] It was shown that HRP also catalyzed oxygenation of p-methylthioanisole at a much lower rate; although the oxygen atom of the sulfoxide product also arose from H_2O_2, equal amounts of both isomers of this sulfoxide were formed.[19] However, HRP-catalyzed sulfoxidation of another compound p-methoxythioanisole resulted in only partial incorporation of oxygen from H_2O_2 and demonstrable incorporation from ^{18}O-labeled H_2O, with no evidence for involvement of molecular O_2 in any of these reactions.[19] In an attempt to explain these somewhat perplexing results in terms of specific mechanisms, Kobayashi et al. carried out studies on a series of *para*-substituted thioanisoles, which demonstrated a qualitative increase in reactivity with increasing electron-donating property of the substituent.[19] From Hammett plots of various rates, it was concluded that the chloroperoxidase-catalyzed reactions were most consistent with direct transfer of an enzyme-bound oxygen atom, presumably from compound I, to the sulfide; there was no evidence for involvement of a free radical species arising from the sulfide in this enzyme system.[19] The possibility certainly exists that the single oxygen atom of chloroperoxidase compound I is sufficiently energetic to oxygenate an arylalkylsulfide which is itself more "activated" for oxidation than Cl^- or styrene. However, an alternative route for formation of chiral sulfoxide products with chloroperoxidase which is analogous to the styrene epoxidation reaction cannot be eliminated: transfer of an electron from the sulfide to compound III to produce a transient hemeprotein-O_2^{2-} intermediate with oxidation state of +5 that immediately transfers an oxygen atom to the immobilized sulfide radical cation. This latter radical might receive the requisite electron for sulfoxide product formation from H_2O_2 either before or after the radical dissociates from the enzyme. Thus, the experimental evidence against participation of a sulfide free radical in the chloroperoxidase-catalyzed reaction does not eliminate the possibility that one or more enzyme-bound radical species derived from the sulfide might participate in the reaction.

Quite different results were obtained for the HRP-catalyzed reactions. Most thianisoles of the series were shown to reduce compound II, with a rate constant that decreased with increasing electron-withdrawing ability of the substituents.[19] Two routes of sulfoxide formation were proposed, each involving the enzymatically produced sulfide radical cation. The enzyme played no further role in one mechanism: the radical cation, once dissociated from the enzyme, could undergo disproportionation with a like species to produce the dication.[19] The sulfide dication could react with H_2O, consistent with a known reaction sequence of sulfide radical species;[154] this would account for partial incorporation of oxygen from H_2O into the product sulfoxide.[154] In order to account for the remainder of the product oxygen arising from H_2O_2, it was proposed that the radical cation species could also undergo reaction with HRP compound II, resulting in transfer of the heme-associated oxygen atom to the radical.[19] This mechanism, if correct, would be the first example of direct oxygen atom transfer from a higher oxidation state of HRP into an *organic* substrate.

An alternative reaction sequence that accounts for incorporation of oxygen from H_2O_2 into the sulfoxide product of *p*-methoxythioanisole in the HRP-H_2O_2 system is suggested. The enzymatically produced sulfide radical cation may react directly with H_2O_2, as another example of reductive activation of H_2O_2 by an organic radical. This reaction is similar to that of Equation 22, proposed for phenylacetaldehyde formation from styrene in the chloroperoxidase-H_2O_2 system:



(23)

The radical cation of the arylalkylsulfide and the styrene radical have similar properties that should facilitate such a reaction: both are electron-rich aromatic species that allow extensive delocalization of the unpaired electron, with an unshared pair of electrons available for donation to an oxygen atom derived from H_2O_2. In the simplest terms, Equations 22 and 23 represent radical-promoted reductive cleavage of H_2O_2 to produce an incipient HO· radical species, which is trapped with high probability at the site of its generation by the organic molecule that supplied the electron.

Sulfoxidation reactions catalyzed by chloroperoxidase and HRP represent one reaction type for which the steric[156-158] and energetic constraints on oxygen atom transfer can be partially resolved. Arylalkylsulfides appear to be the only sulfides converted to sulfoxides by a chloroperoxidase-H_2O_2 system in the absence of halide.[19] The conversion of methionine to methionine sulfoxide (racemic) by chloroperoxidase requires both H_2O_2 *and* halide anion.[73] Since such conditions would result in halide oxidation, and since the electrons of methionine are less activated than those of arylalkylsulfides, the sulfoxide product isomers are likely formed by a nonenzymatic reaction between the oxidized halogen species and methionine. We note that HRP can catalyze the H_2O_2-dependent oxygenation of arylalkylsulfides in the absence of an oxidizable "cosubstrate" but requires a "cosubstrate" to form styrene oxide from styrene. Since these sulfides are relatively more activated for one-electron oxidation than styrene, this result provides at least indirect support for participation of a radical species of the oxygen acceptor in the overall reaction. Transfer of an oxygen atom by oxidized hemeprotein species in a catalytically efficient manner appears to require close coupling of the electron transfer and oxygen atom transfer component reactions. This coupling would necessarily impose both energetic and steric requirements on the enzyme active site and transition states. The aromatic character of the arylalkylsulfide likely facilitates its one-electron oxidation by HRP, but hinders the stereospecific transfer of a heme-associated oxygen atom to the substrate sulfur atom, if indeed this were energetically possible for HRP. As a consequence, electron abstraction from the substrate becomes uncoupled from oxygen atom transfer: HRP can directly catalyze the former reaction but not the latter, which was proposed to occur by a nonenzymatic reaction of the sulfide radical with H_2O_2. Thus, HRP is a very poor catalyst of sulfoxide formation.[19] By contrast, arylalkylsulfides appear to satisfy both energetic and steric constraints of chloroperoxidase, with respect to efficient coupling of the electron abstraction and oxygen atom transfer component reactions associated with the oxygenation.[19] The failure of chloroperoxidase to oxygenate methionine, a dialkylsulfide, with H_2O_2 alone appears to be a specific example for which both redox and steric factors may prevent the direct reaction of the sulfide with chloroperoxidase compound III.

The reactions depicted by Equations 22 and 23 may occur more generally during hemeprotein-catalyzed oxidation of aromatic compounds than has been recognized, whether the oxidant is H_2O_2 or an organic hydroperoxide. If O_2 can give rise to H_2O_2 in a given system, then the

incorporation of oxygen from O_2 into one or more products could occur by Equation 23. Thus, experiments with ^{18}O-labeled O_2, H_2O_2, and H_2O are necessary to determine unequivocally the origin of oxygen in products of enzyme-mediated radical oxidation reactions involving peroxides and O_2. However, establishing the origin of oxygen in the product(s) does not necessarily identify the nature and/or oxidation state of the oxidant, especially if reactions given by Equation 22 and Equation 23 can occur. This caveat complements earlier comments about the difficulty of defining *the* mechanism of a very complex enzyme-initiated free radical reaction from single turnover experiments. The most favorable enzymatic and nonenzymatic reactions in very similar systems may vary, depending upon the initial experimental conditions, as well as changes in composition of the reaction mixture as the reaction progresses through the distinct initiation, steady-state, and termination phases characteristic of radical reactions.

V. NONHEME HALOPEROXIDASES

An in-depth review of other haloperoxidases is beyond the scope of this review; other chapters in this series deal with selected hemeprotein haloperoxidases. Where appropriate, comparisons of chloroperoxidase structure or catalytic function with those of other selected hemeproteins have been made. A comprehensive, well-organized survey of the extraordinarily diverse sources of haloperoxidases in nature was provided in the book by Neidleman and Geigert.[2] However, only two nonheme haloperoxidases will be briefly discussed to reinforce a conclusion that can be inferred from the material presented in this and other recent reports[2,9,159,160] on enzymatic halogenation reactions: that nature has employed diverse protein structures, different prosthetic groups, and most likely some subtle differences in catalytic mechanism to accomplish the same end, i.e., the halogenation of organic compounds.

The group at Cetus Corporation has recently described the properties of the first reported nonheme chloroperoxidase, purified from the fermentation broth of another fungus *Curvularia inaequalis*.[9] The enzyme, with molecular weight of about 240,000 Da, is a tetramer, and each subunit has the same molecular weight, 66,000 Da. No evidence for a heme prosthetic group was found, but X-ray fluorescence data revealed the presence of 2.2 atoms of zinc and 0.7 atoms of iron per enzyme molecule.[9] The carbohydrate content of the enzyme was determined to be 9%. The enzyme could catalyze chlorination of MCD and allyl chloride, and could utilize Br^- and I^- (but not F^-), in addition to Cl^-.[9] Under the same experimental conditions, the pH optimum for MCD chlorination by both chloroperoxidase enzymes was the same, but the catalytic activity of the *C. fumago* enzyme was about six times greater. Under conditions where the *C. fumago* chloroperoxidase catalyzes epoxidation of alkenes or peroxidation of primary alcohols to aldehydes, the nonheme enzyme did not display either activity. Of considerable interest was the finding that the nonheme chloroperoxidase was considerably more stable than its *C. fumago* counterpart upon exposure to moderate concentrations of HOCl or high concentrations of H_2O_2.[9] Although no attempt was made to define the catalytic cycle of the nonheme chloroperoxidase from *C. inaequalis*, it is apparent from this report that a heme group is not required for competent catalysis of H2O2-dependent Cl^- oxidation by an enzyme, and that the heme group of the *C. fumago* chloroperoxidase confers a greater range of reactivity, with consequent advantages and disadvantages to this enzyme.[9]

Finally, mention is made of a vanadium-containing bromoperoxidase from *Ascophyllum nodosum*,[160] one member of a class of nonheme bromoperoxidases from various marine sources, which have been extensively characterized by Wever and colleagues.[161,162] In a recently published steady-state kinetic analysis of MCD bromination by this enzyme, de Boer and Wever proposed a minimal catalytic cycle in which the enzyme binds H_2O_2 and then Br^- to form a ternary complex which decays to native enzyme and the product HOBr.[160] The similarities between bromination reactions catalyzed by this vanadium-containing bromoperoxidase and quite generally by heme-containing haloperoxidases suggest that specific constraints on halide

oxidation by H_2O_2, which we believe to be primarily thermodynamic in nature, dictate certain minimal requirements for any catalyst of these reactions. The data provided strong evidence that the *A. nodosum* enzyme produces free HOBr, which functions as the chemical brominating agent. Also, the rates of bromination of several compounds, including MCD, were very comparable, and related to the degree of pi electron density on the molecules,[160] a measure of hydrogen atom activation. Indeed, the same compounds are efficiently halogenated by *C. fumago* chloroperoxidase and by *A. nodosum* bromoperoxidase.

The data required an ordered binding of substrates to the enzyme, H_2O_2 first, followed by Br^-, suggesting that the O-O bond of enzyme-bound H_2O_2 may be cleaved only after Br^- binds to the enzyme.[160] The pH dependence of Br^- binding suggested that the halide binds preferentially to a more acidic enzyme-H_2O_2 complex, but can also bind to other protonation states of this complex in a productive manner to yield HOBr.[160] The results of this study are consistent with, but do not prove, the idea that an H_2O_2 species with a distinct O-O bond bound to the active site of the enzyme may be required for productive oxidation of Br^-.[160] If this inference about mechanism is correct, it would imply that the energy needed to form an O-Br^- bond requires a vanadium-bound peroxidic species, quite analogous to the proposal for Cl^- oxidation by the heme-containing chloroperoxidase. For the nonheme containing haloperoxidases, which utilize either iron or vanadium as the active redox center, discrete oxidation states of the metal ion functional in the catalytic cycle have not yet been identified. Because of the absence of strong absorbance bands associated with the metal ions, defining the catalytic cycles of the nonheme haloperoxidases will be experimentally more difficult than for the hemeprotein haloperoxidases.

VI. CONCLUSION

In this review of the hemeprotein chloroperoxidase from *C. fumago*, an attempt has been made to analyze critically and objectively published data on the catalytic functions of this enzyme, and to integrate these data into a working hypothesis that can account for the characteristic Cl^- oxygenation activity of this enzyme. There is no direct evidence demonstrating that Cl^- oxidation by this enzyme occurs by the currently accepted mechanism of halide oxidation catalyzed by other peroxidases. Since certain other findings are also not consistent with the standard mechanism, a hypothesis has been proposed in which compound III and a one-electron oxidized radical species of the terminal halogen acceptor play critical roles in the Cl^- oxygenation and subsequent chlorination activities of chloroperoxidase.

Arguments for the obligatory involvement of compound III of chloroperoxidase in the activation of an oxygen atom for transfer to certain acceptor molecules are at this time indirect. Compound III has been characterized by several laboratories;[133-135] it is very reactive under experimental conditions quite different from optimal conditions for halide oxidation, suggesting that it would be even more reactive under optimal halogenation conditions. However, critical experimental tests of this hypothesis about the role of compound III in the catalytic function of chloroperoxidase have not been reported. The only published data which support indirectly this idea relate to myeloperoxidase function: Winterbourn et al.[150] showed that myeloperoxidase exists predominantly as its compound III species during active phagocytosis of neutrophils. The lack of definitive experimental data related to chloroperoxidase reaction mechanism is likely a direct consequence of the difficulty of correlating data obtained from single-turnover experiments with data generated under catalytic conditions. Because chloroperoxidase has a very large catalytic activity, the two kinds of experiments must be performed under quite different experimental conditions, which can alter dramatically the course of mixed enzymatic/radical chain reactions as complex as those involving chloroperoxidase.

Two lines of reasoning support the proposed roles of compound III and radical species of the terminal halogen acceptor in oxygenation reactions of chloroperoxidase. The first argument is

based on the thermodynamics of Cl^- oxidation by H_2O_2: under standard conditions, formation of the O-Cl^- bond would require a considerable fraction of the energy available from reduction of H_2O_2. A three-electron reduction of compound III from a +6 to a +3 oxidation state, requiring an electron from an exogenous source, would yield more energy than a two-electron reduction of compound III or compound I. Compound I species contain only one oxygen atom, and only a part of the total energy, originally available in the peroxidic substrate. Thus, electron transfer reactions of compound I appear to be restricted to easily oxidized substances. There is only limited indirect evidence suggesting that HRP compound I may transfer an oxygen atom to a few compounds, which include I^- and Br^-; there appear to be stringent thermodynamic *and* steric[18,156-158] constraints quite generally on oxygen atom transfer reactions of this hemeprotein. An analysis of Br^- oxidation by H_2O_2 catalyzed by HRP suggested that the negative ΔG value of the overall reaction must be apportioned among the individual reactions of the catalytic cycle, so that *each* reaction, especially the last one, has a negative ΔG, to insure irreversibility of the overall reaction. The implication of this result is that the energy requirement of the Cl^- oxidation step will include not only the energy needed to form the O-Cl^- bond but also an additional amount of energy, appearing as a negative ΔG, so that the product-forming last step in the cycle is thermodynamically favorable. Transfer of an electron to a compound III with a bound O_2 species would generate *in situ* a heme-coordinated peroxide of unknown protonation state. The formal oxidation state of this complex would be +5, but it would contain both atoms of the oxidant; for this reason, this complex is considered to be more reactive than a compound I species, which has the same formal oxidation state, but a quite different distribution of electrons. The reactivity of this transient activated heme-bound peroxidic species appears to be that of an oxygen transfer agent. The immediate environment of the species, determined by both the protein and the acceptor molecule, would dictate the reaction path leading to final products.

Finally, the hypothesis for a critical function of compound III in chloroperoxidase-catalyzed oxygenation reactions provides a functional rationalization of the similar heme structures of this enzyme and cytochromes P-450. The dioxygen-ferrous-substrate complex of cytochrome P-450, which is a resonance form of compound III, is acknowledged to be the requisite intermediate on the direct path of O_2 activation for hydrocarbon oxygenation. Indeed, the catalytic cycle for Cl^- oxidation by H_2O_2 involving compound III of chloroperoxidase can be formally considered as a monooxygenation reaction; two molecules of the halogen acceptor serve the electron donor function that NADPH performs in cytochrome P-450 monooxygenation reactions. However, an important distinction between the two hemeproteins is that some isozymes of cytochrome P-450 are capable of inserting an oxygen atom into aliphatic hydrocarbons, whereas chlorination reactions (and probably oxygenation reactions as well) of chloroperoxidase appear to require some degree of activation of hydrogen atoms or electrons in the acceptor molecules. Thus, cytochrome P-450 appears capable of generating a more energetic oxygen atom from reductive cleavage of heme-bound O_2 than chloroperoxidase. This difference is attributed, in part, to a very hydrophobic protein active site environment of cytochrome P-450, which not only binds the hydrophobic substrate in a specific manner, but also limits access of solvent H_2O to the heme complex during the critical oxygen activation step.[33,38]

In summary, a working hypothesis has been proposed for the reaction mechanism of chloroperoxidase oxygenation activity, which is generally applicable to Cl^- and certain organic compounds as oxygen acceptors. The essential role of compound III in these reactions, as proposed, links the function of this hemeprotein to the function of cytochromes P-450 and other hemeproteins that bind O_2 during their physiologic functions. The diverse reactivities of compound III species suggest that such oxidized heme species, rather than compound I-like species, may play important, but previously unrecognized, regulatory roles in the catalytic cycles of many hemeproteins. Perhaps compound III species provide an evolutionary link between a primitive reactive heme-O_2 complex and hemeproteins with remarkably diverse functions. The potential importance of compound III species in the catalytic function of

chloroperoxidase, as proposed in this review, and in the catalytic functions of certain other hemeproteins, in their physiologic environments, requires further study.

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In this attempt to relate the structure and function of *C. fumago* chloroperoxidase to hemeproteins of the peroxidase and cytochrome P-450 classes, it became necessary to limit the references cited from the large number of publications which have appeared in these three major research areas. Since the selection of references involved some unintentional bias, apologies are made to those scientists whose contributions may not have been adequately acknowledged. I express sincere appreciation to my highly supportive husband Jim, who relinquished his time share on our home computer to accommodate my writing this review. Finally, I would like to acknowledge coworkers in my laboratory, colleagues with whom I have been associated, and many distinguished scientists in the area of hemeprotein structure and function whose ideas and contributions directly influenced the ideas expressed in this review. The pioneering work of Dr. Isao Yamazaki in elucidating remarkably complex kinetics of peroxidase-catalyzed reactions is acknowledged with great admiration and respect.

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